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Full production cycle performance of gene-edited, sterile Atlantic salmon - growth, smoltification, welfare indicators and fillet composition

L. Kleppe [a,*], P.G. Fjelldal [b], E. Andersson [a], T. Hansen [b], M. Sanden [a], A. Bruvik [a], K.O. Skaftnesmo [a], T. Furmanek [a], E. Kjærner-Semb [a], D. Crespo [a], S. Flavell [b], A.Ø. Pedersen [b], P. Vogelsang [a], A. Torsvik [a], K.A. Kvestad [b], S. Olausson [c], B. Norberg [c], R.W. Schulz [a,d], J. Bogerd [d], N. Santi [e], R.B. Edvardsen [a], A. Wargelius [a]

[a] Institute of Marine Research, P.O. Box 1870, Nordnes, NO-5817 Bergen, Norway
[b] Institute of Marine Research, Matre Research Station, NO-5984 Matredal, Norway
[c] Institute of Marine Research, Austevoll Research Station, NO-5392 Storebø, Norway
[d] Utrecht University, Science Faculty, Department Biology, Padualaan 8, NL-3584, CH, Utrecht, Netherlands
[e] AquaGen AS, P.O. Box 1240, Torgard, NO-7462 Trondheim, Norway

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ABSTRACT

Using germ cell-free (GCF), sterile, *dnd*-knockout salmon for farming could solve the problems associated with precocious maturation and genetic introgression of farmed breeds into wild populations. However, prior to using GCF fish in the salmon farming industry, it is crucial to understand if, or how, the GCF phenotype differs from wild type (WT) counterparts in terms of growth and welfare. To characterize the GCF phenotype throughout a production cycle, we reared GCF and WT salmon in indoor common garden tanks for 3 years, until harvest size. Regarding body size, smoltification markers (mRNA levels of gill Na^+/K^+-ATPase [NKA] subunits), plasma stress indicators (pH, glucose, sodium, chloride, calcium), relative heart size, prevalence of vertebra deformities and fillet proximate composition, GCF fish could not be distinguished from WTs. Transient differences were detected in plasma concentrations of lactate and osmolality, and only a few genes were differentially expressed in WT and GCF transcriptomes of muscle and pituitary. At harvest, fillets from GCF and WT salmon contained the same amount of omega-3 fatty acids, however the relative content of omega-3 fatty acids was higher in GCF compared to WT males. Towards harvest size, body growth rate, condition factor and relative liver size were significantly higher in WT than in GCF fish, probably relating to initiation of puberty in WTs. Since GCF salmon never become sexually mature, it is possible to postpone the time of harvest to exploit the growth potential uninhibited by sexual maturation. In conclusion, GCF salmon performed to a large extent similarly to their WT counterparts but had the clear advantage of never maturing.

1. Introduction

Atlantic salmon (*Salmo salar*) farming in open sea cages is a key industry in Norway. However, a high density of salmon farms along the coastline also represents a risk for negative environmental impacts. One major concern is related to potential effects on wild populations conveyed by escapees from salmon farms. Interbreeding of farmed and wild salmon can result in genetic introgression (Glover et al., 2012), which may in turn affect biological characteristics of the wild stocks (Bolstad et al., 2017; Glover et al., 2017). One potential solution is to block the ability to reproduce by inhibiting the function of proteins that are important for germ cell development and/or survival (Wong and Zohar, 2015a). We have previously produced a germ cell-free (GCF), and hence sterile, salmon model by knocking out the *dead end* (*dnd*) gene (Wargelius et al., 2016). Moreover, this fish could not be triggered into sexual maturation using an established maturation-inducing rearing regime (Kleppe et al., 2017). In addition, GCF salmon may potentially be produced at a large scale using knockdown technology (Wong and Zohar, 2015b) or germ cell rescue in genetically sterile broodstock (Güralp et al., 2020). This is a promising trait with the potential to solve the issue with interbreeding between farmed and wild salmon, as well as early unwanted sexual maturation known to negatively affect fish

* Corresponding author.
E-mail address: lene.kleppe@hi.no (L. Kleppe).

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welfare (reviewed by Taranger et al., 2010). While gene editing of farmed animals requires an extensive process of approval in Norway, the currently used method for production of sterile farmed salmon is triploid induction (Benfey, 2016). This technique is not widely adopted by the industry since farmed triploid salmon show welfare problems and higher production costs. For example, triploid salmon are more likely to develop skeletal deformities than their diploid counterparts (Fjelldal and Hansen, 2010; Fraser et al., 2013; Fraser et al., 2015; Amoroso et al., 2016a; Amoroso et al., 2016b). Skeletal deformities represent a significant metabolic cost and may affect swimming performance (Powell et al., 2009) and growth in Atlantic salmon (Toften and Jobling, 1996; Hansen et al., 2010). In general, triploid fish (across different species) seem more prone to develop deformities, and they are more susceptible to thermal stress than diploids (reviewed by Fraser et al., 2012). Therefore, there is a need for alternative sterility models.

Although loss-of-function gene editing is currently not being used in the Norwegian fish farming industry, other countries such as Japan (Nature Biotechnology, 2022) and Argentina are now using edited fish species, displaying targeted loss-of-function mutations in their genomes, for human consumption. Furthermore, numerous studies are being performed globally on gene editing of different farmed fish species, focusing on production traits such as reproduction, growth and disease resistance (Blix et al., 2021; Gratacap et al., 2019; Wargelius, 2019). Therefore, it appears necessary to perform a careful assessment of potential effects of removing *dnd* gene function and the resulting lack of germ cells, on salmon welfare and production traits, as well as effects on the product quality for the consumers. For example, the comparison of edible tissues from wild type (WT) versus GCF salmon will add valuable information to the process of assessing food quality. Exploring the sustainability aspects of this alternative sterility model will provide the information required for evaluating the potential future use of such models in aquaculture.

When assessing the performance and welfare of a farmed animal, it is crucial to keep in mind that each species and life stage have different requirements. In the case of farmed Atlantic salmon, an overview of measurable welfare indicators was recently published (Noble et al., 2018). Evidently, the higher the number of welfare indicators measured, the more precise the assessment will be. One such welfare indicator is body growth, which has a clear link to feeding and nutritional needs, but may also serve as indicator of other welfare issues like deformities, disease or poor water quality. Proper smolt quality and development of hypo-osmoregulatory ability during smoltification is crucial for anadromous Atlantic salmon that move from freshwater (FW) to seawater (SW), and can be determined by measuring the activity of the sodium potassium ATPase (NKA) enzyme in their gills (Nilsen et al., 2007). Changes in plasma osmolality (the number of dissolved particles in liquid), concentration of ions (sodium, chloride and calcium), pH and metabolites (glucose and lactate) may indicate stress. Reduced bone health by skeletal deformities, as indicated above, are associated with reduced welfare. Abnormal abdominal organs are often indicative of a disease condition. Sexual maturation can cause problems with immune capacity, osmoregulation and aggressive behavior, as well as increased mortality (Taranger et al., 2010) (for a detailed overview of known welfare indicators in Atlantic salmon, see Noble et al., 2018).

The overall findings in this study, after following GCF and WT salmon through a complete production cycle, are as follows: 1) GCF and WT salmon could not be distinguished with regards to body size, smoltification markers, several plasma stress indicators, relative heart size, prevalence of vertebra deformities or fillet proximate composition, 2) transient differences were detected in plasma concentration of lactate and osmolality, 3) comparison of GCF and WT muscle and pituitary transcriptomes revealed none or very few differentially expressed genes, 4) fillets from GCF salmon displayed the same amount of omega-3 fatty acids as fillets from WTs, however the composition was different in GCF males (higher relative omega-3 proportion than WT males), 5) towards harvest size, WT fish had higher body growth rate and condition factor, and larger relative liver size compared to the GCF group, in line with the onset of puberty (which was not detected in GCF fish). Sterile, GCF salmon may be applied in future aquaculture to avoid genetic introgression with wild populations, prevent maturation-associated welfare problems, protect the intellectual property rights of breeding companies, and produce larger fillets.

2. Methods

2.1. Ethical statement

The use of the experimental animals in this study was performed in accordance with the Norwegian Animal Welfare Act of 19th of June 2009, in force from 1st of January 2010. All the fish reared were approved by the Norwegian Animal Research Authority (http://www.fdu.no/fdu/NARA, permit number 5741).

2.2. Experimental animals and setup

Fish rearing and the experiment took place at the Institute of Marine Research, Matre Research Station, Norway. In October–December 2016, Atlantic salmon embryos (freshly fertilized eggs) were genome edited using CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats and CRISPR-associated protein 9) to induce knockout (KO) of the *dnd* gene, subsequently causing lack of germ cells and sterility as described previously (Wargelius et al., 2016). Briefly, we designed and synthesized a guide RNA targeting *dnd*, and injected it into the eggs together with *cas9* RNA. Within the cell, the guide RNA directed the Cas9 protein to make a double-stranded cut in *dnd*. The resulting mutation caused a non-functional *dnd* gene. In addition to *dnd*, we also induced knockout of *slc45a2*, which causes lack of pigmentation and is used as a screening tool for KO of the target (*dnd*) gene (Wargelius et al., 2016). In August 2017, all fish (n = 242) were placed in a common garden setup; *dnd*-KO (n = 94) were reared together with non-injected WT individuals (n = 148). The fish were reared in indoor tanks with FW and later SW (after smoltification), natural light conditions, ambient temperature and fed ad libitum with a standard commercial salmon diet until February 2020. The feeds were obtained from Skretting AS (Sjøhagen 15, 4016 Stavanger, Norway), with pellet size adjusted to fish size throughout the study period. For an overview of the experimental setup see Table A.1.

The total number of fish in this study was originally 434 (179 *dnd*-KO and 255 WT). However, 43 (24.0%) *dnd*-KO and 85 (33.3%) WT fish died during the experiment and are therefore not included. This was mainly caused by a technical incident in one fish tank, which explains the loss of 27 and 53 of the dead *dnd*-KO and WT individuals, respectively. The remaining mortality of 16 *dnd*-KO (8.9%) and 32 WT (12.5%) was due to unknown factors and distributed throughout the different life stages. In addition to the fish that died, the following were not included in this study: 1) 20 *dnd*-KO and 21 WT to be used in a separate experiment, 2) 6 *dnd*-KO that smoltified one year later than the rest of the fish (see Section 3.4), 3) 15 partial *dnd*-KO that contained germ cells (see Section 3.2), 4) 1 partial *dnd*-KO sampled for RNA-sequencing (RNA-seq) that contained germ cells, 5) 1 WT that had lost the pit-tag.

2.3. Samplings

Prior to all samplings of length, weight, X-rays and gill biopsies, the fish were anesthetized with 100 mg/L Finquel vet. (tricaine methanesulfonate), and at larger fish sizes clove oil (1 mL/100 L Aqui-S vet.) was applied as a sedative before measuring length and weight. Prior to all end-point samplings, the fish were anesthetized with 1 g/L Finquel vet., and sacrificed by cutting the medulla oblongata. Body length and weight were measured for all fish four times each year, starting in November 2017. At this first sampling time, all fish were also pit-tagged, fin-clipped and X-rayed. Four months prior to, and 24 h after transfer to

SW, gill biopsies were collected from 8 *dnd*-KO and 8 WT fish. From the same individuals, blood was collected 24 h after transfer to SW. Six months after transfer to SW the following samples were collected from 23 fish (6 *dnd*-KO males, 6 *dnd*-KO females, 5 WT males and 6 WT females): pituitary and muscle tissue, blood and vertebrae. Also, the heart and liver weights were measured. In November 2018, all fish were X-rayed again. Blood samples were centrifuged at 14,000 x *g* in 4 °C for 2 min, plasma was collected and stored in −80 °C until further analyses. Tissue samples were stored in RNA later (ThermoFisher Scientific) at 4 °C or − 80 °C until further use. Vertebrae samples were stored at −20 °C until further analyses. At the end of the experiment, the remaining fish (n = 219) were sacrificed and the following was collected/measured from all individuals: body length and weight, heart-, liver- and gonad-weight, blood and X-ray. Furthermore, tissues for histology, gene expression and mutation analyses as well as whole skin-free fillets of individual salmon for fillet fatty acid composition were collected from a subset of fish (for a detailed overview of all sampling during the study see Table A.1).

2.4. Analysis of plasma biochemical markers

All plasma samples were analysed for osmolality using a Fiske™ 210 Micro-Sample Osmometer (Advanced Instruments). For postsmolts after 24 h in SW, plasma Na^+ and Cl^- were measured using an AVL 9180 Series Electrolyte Analyser (Roche Diagnostics). Plasma lactate was measured using a Maxmat PL biochemistry analyser. Plasma pH was measured using a pHM 92 pHmeter (Radiometer). For plasma samples from harvest size fish, the following parameters were measured using an ABL 90 Flex plus (Radiometer): pH, lactate, glucose, Na^+, Ca^{2+} and Cl^-.

2.5. Analysis of vertebra health

Radiographs of whole parr and postsmolts were taken with a Computed Radiography system (CR35 VET, DÜRR NDT GmbH & Co. KG, Bietigheim-Bissingen, Germany) using a 35 × 43 cm image plate (IP 35/43, DÜRR NDT GmbH & Co. KG, Bietigheim-Bissingen, Germany) and a portable X-ray unit (Portable X-ray Unit Hiray Plus, Model Porta 100 HF, JOB Corporation, Yokohama, Japan) at 85 cm distance with 40 kV and 10 mAs. For harvest size fish, we applied a Direct Radiology System (Canon CXDI-410C Wireless, CANON INC., Kawasaki, Japan) using a portable X-ray unit (Portable X-ray Unit Hiray Plus, Model Porta 100 HF, JOB Corporation, Yokohama, Japan) at 85 cm distance with 40 kV and 4 mAs.

For mechanical testing, vertebrae nos. 40 to 43 were carefully dissected from each specimen and had their neural and haemal arches and remains of notochordal tissue removed. Following dissection, the vertebrae centra were tested with a texture analyser (Model: TA-HD plus Texture Analyser, Stable Micro Systems Ltd., Surrey, UK) by compressing single vertebral centrums along their cranio-caudal axis with a steadily advancing piston (0.01 mm/s), and the stiffness calculated for each vertebra according to Fjelldal et al., 2004. Following mechanical testing, the vertebrae from each fish were pooled and defatted in a 2:1 volume mix of acetone and chloroform for 24 h, dried overnight at 100 °C and incinerated for 10.5 h in a muffle furnace (Mod. L 40/11/P320; Nabertherm GmbH) (115 °C for 0.5 h, 540 °C for 4 h and 750 °C for 6 h), according to Kacem et al., 2000. The dry weight and total ash weight of each pooled sample was measured to the nearest 10^{-2} mg. The total ash content was calculated as follows: total ash content (% dry weight) = (total ash weight x 100) x (dry weight)$^{-1}$.

2.6. Analysis of plasma steroid hormone levels

The sex steroids estradiol-17β (E_2), 11-ketotestosterone (11-KT) and testosterone (T) were extracted from blood plasma by a method modified from Pankhurst and Carragher, 1992. The extracted and dissolved steroids were stored at −20 °C until analysis by an enzyme-linked immunosorbent assay (Cuisset et al., 1994), previously validated for Atlantic salmon (Andersson et al., 2013).

2.7. DNA extraction and sequencing

DNA from fin and gonad tissue was isolated using DNeasy 96 Blood & tissue kit (Qiagen), according to the manufacturer's instructions. The sex of each fish was determined using *sexually dimorphic on the Y-chromosome* (*sdY*) genotyping with qPCR (Ayllon et al., 2019). *dnd* mutation analysis was performed using MiSeq (Illumina), as described previously (Güralp et al., 2020).

2.8. RNA extraction

For the smoltification marker analysis, total RNA was extracted from 2 gill lamellae using the RNeasy micro kit (Qiagen). The RNA was DNase treated as part of the RNA extraction protocol. RNA concentration and purity were measured using a Nanodrop spectrophotometer (ThermoFisher Scientific), all samples showing absorbance ratios 260/280 of 2.0–2.4.

For the RNA-seq analysis of pituitary and muscle tissue, and the qPCR for *vasa* screening in the gonads, total RNA was extracted from whole pituitaries or approximately 3 mm^3 muscle/gonad tissue using the Maxwell HT simplyRNA kit (Promega), according to the manufacturer's instructions. A BioMek 4000 instrument (Beckton Dickinson) was applied for the RNA extraction, and RNA was DNase treated as part of the RNA extraction procedure. RNA concentration and quality were measured using a Nanodrop spectrophotometer (ThermoFisher Scientific), all samples showing absorbance ratios 260/280 of 1.8–2.2. Furthermore, all samples for RNA-seq were checked by a Bioanalyzer (Agilent Technologies), showing RNA integrity numbers of 6–10.

2.9. RNA-seq

TruSeq Stranded mRNA libraries were prepared from muscle and pituitary samples from 11 WT and 12 GCF fish and sequenced using the HiSeq4000 sequencing platform (Illumina). The Paired-End 2x75bp sequencing gave on average of 52,4 million reads pr. sample (raw sequence reads accession no. PRJNA764865; https://www.ncbi.nlm.nih.gov/bioproject/PRJNA764865).

RNA-seq paired end sequences were mapped with Bowtie2 against the gene model transcripts of Atlantic salmon genome (ICSASG_v2) with standard Bowtie2 parameters (Langmead and Salzberg, 2012). Raw count table for each gene was extracted using SAMtools idxstats (Li et al., 2009). The read counts were normalized to the total reads in the sample with the smallest number of reads. Transcripts with <10 reads in all samples were excluded from further analysis. Differential expression analysis was performed using the R/Bioconductor package NOISeq (1-PNOI <0.05, equivalent to false discovery rate (FDR) adjusted *P*-value (Tarazona et al., 2011; Tarazona et al., 2015). Differentially expressed genes with a low average number of reads (<50) in both WT and GCF groups, were considered as background noise and were therefore excluded.

2.10. cDNA synthesis and qPCR

cDNA was synthesized from 250 ng gill or gonad RNA using a VILO cDNA synthesis kit (Invitrogen). For the smoltification marker analysis, qPCRs were performed using primers and probes for the different *nka* subunits (Nilsen et al., 2007). *elongation factor 1 alpha* (*ef1a*) (Olsvik et al., 2005) was used as the reference/housekeeping gene. qPCRs were performed in duplicates in 384-well optical plates in a QuantStudio 5 Real-Time PCR system (ThermoFisher Scientific) using default settings. 2 μl of cDNA (diluted 1:20) was used in 6 μl Fast TaqMan mastermixes. No-template controls for each gene were run in all qPCR plates. The ΔΔCt method was used to calculate the expression of each gene relative

to *ef1a*. For each gene, all expression values were calibrated to the mean ΔCt of the WT fish group at the first sampling point (pre-smolt). For the *vasa* screening analysis, previously published primers and probe were applied (Wargelius et al., 2016). qPCRs were performed as described above for the *nka* subunits, with minor changes. Briefly, cDNA diluted 1:80 was used, and all expression values were calibrated to the WT sample with the lowest ΔCt (highest expression).

2.11. Fillet proximate analysis and fatty acid composition

Homogenates from whole skin-free fillets (a standardized muscle sample, Norwegian Quality Cut, as described by Johnsen et al., 2011) of individual salmon were analysed for protein, total lipid, dry matter and fatty acid composition. Nitrogen (N) was measured with a nitrogen analyser (Vario Macro Cube) according to AOAC, 2005, and protein was calculated as N x 6.25. Total lipid of the fish fillets was measured gravimetrically after ethyl acetate extraction and after acid hydrolysis for the feed samples. Dry matter was measured gravimetrically after freeze drying for 48 h. Fatty acids were analysed as described previously (Lundebye et al., 2017). Briefly, fatty acids were analysed on a HP-7890A gas chromatograph using a flame ionization detector (GC-FID), and with nonadecanoic acid (19:0) as an internal standard. The fatty acids methyl esters (FAME) were extracted using hexane. The fatty acids were identified by retention time in combination with FAME standards and a fatty alcohol standard. Chromatographic peak areas were corrected by empirical response factors calculated from the areas of the GLC-463 mixture. The chromatograms were integrated using EZChrom Elite software from Agilent Technologies.

2.12. Calculations

Condition factor (CF) = (100 x body weight (g)) / fork length (cm) ^3).

Specific growth rate (SGR) = (ln(body weight2 (g)) – ln(body weight1 (g)) / n x 100, where body weights 1 and 2 correspond to time point 1 and 2; n indicates the number of days from time point 1 to 2.

Fork length daily growth (mm/day) = (fork length2 (cm) – fork length1 (cm)) / n x 10, where fork lengths 1 and 2 correspond to time point 1 and 2; n indicates the number of days from time point 1 to 2.

Gonado/hepato/cardiosomatic index (GSI/HSI/CSI) = (organ weight (g) / body weight (g)) x 100.

2.13. Statistics

All statistical tests were performed using GraphPad Prism version 9.0.0 (GraphPad Software, La Jolla California USA, www.graphpad.com). All datasets were tested for normal distribution using a D'Agostino & Pearson normality test. Parametric tests were applied in all cases where the datasets to be compared passed the normality test. Non-parametric tests were used for datasets without normal distribution or with too low n ($n < 8$) to test for normality. For multiple comparisons (including all four groups of fish; WT females, GCF females, WT males and GCF males), a One-way ANOVA with Tukey's multiple comparisons test or Kruskal-Wallis test with Dunn's multiple comparisons test were applied. In cases with low n ($n < 8$), an additional Mann-Whitney test was used to compare WT with GCF fish of the same sex, excluding the other two groups of fish, to detect significant differences that the multiple comparison could not detect. Regarding prevalence of vertebra deformities, a Fisher's exact test was applied to test for significant difference between WT and GCF fish. In this case, we combined females and males due to unfulfilled conditions for performing Chi-square calculations. The test(s) used for each dataset is included in the corresponding figure or table legend.

3. Results

3.1. Experimental system

In this study we measured performance of *dnd*-KO, GCF and hence sterile, Atlantic salmon over 3 years, from the parr stage until adults of a typical harvest size in commercial salmon farming. We used the term "postsmolts" for salmon that had recently been transferred to SW, lasting until the end of the same year. An overview of all parameters measured is shown in Fig. 1 and in more detail in Table A.1.

3.2. Lack of germ cells and dnd mutation rate

When knocking out *dnd* in the F0 generation of Atlantic salmon, most individuals completely lack germ cells, which was also the case in this study (see below). However, a small proportion of the fish may have mosaic gonads, where some areas contain germ cells (Wargelius et al., 2016). Here, we observed some individuals with "normal looking" areas of the gonad, and histological investigation and analysis of *vasa* expression (a germ cell marker) (Olsen et al., 1997; Nagasawa et al., 2013) confirmed the presence of germ cells in these individuals. An example of WT vs. GCF gonads with regards to gross morphology and histology is shown in Fig. A.1. In total 15 (9 males, 6 females) out of 103 *dnd*-KO fish had gonads that were fully or partially resembling WT gonads macroscopically. Microscopic analyses confirmed the presence of germ cells, which was supported by expression of *vasa* at levels similar to WT gonads (Fig. A.2). These 15 fish were excluded from further analyses. All *dnd*-KO fish that were considered as GCF at sampling based on the gonad gross morphology were analysed for *vasa* expression, showing all these individuals to have close to zero *vasa* expression in gonad tissue (Fig. A.2).

To confirm the *dnd* mutation efficiency and the corresponding lack of germ cells in the salmon used in this study, we sequenced DNA from gonad and fat fin tissue from a selection of *dnd*-KO and WT individuals, and compared the sequencing results with gonad gross morphology and gonad expression of *vasa*. In each fish where we noted that the gonad looked thin (and therefore GCF) at sampling (fish 1, 3–5, 9, 11, 16, 18, 20, 22, 24, 27), *vasa* expression levels were close to zero (Table 1). Furthermore, these individuals were complete knockouts as shown by 0% (or almost 0%) *dnd* WT sequence in both gonad and fin tissue (Table 1). In contrast, WT fish (individuals 10 and 23) had normal gonad size and *vasa* expression, and 100% *dnd* WT sequence in gonad and fin tissues. In cases where *dnd*-KO fish had a "normal looking" gonad, analysis of *vasa* expression confirmed the presence of germ cells (individuals 12 and 21, Table 1). The presence of germ cells in these two animals was reflected by a lower mutation success (17, 27 and 31, 67% *dnd* WT sequence in fin and gonad tissues, respectively).

3.3. Similar body size in GCF and WT parr to adults at harvest size, but higher growth rate and condition factor in WTs towards harvest size

Body weight and fork length were measured continuously throughout the study as shown in Table 2. For our analyses, WT and GCF fish were sub-divided according to their sex in order to detect possible sex-related differences in the parameters measured. No difference in body weight was detected at any time point, when comparing WT vs. GCF of the same sex, or comparing females vs. males of the same genotype.

Specific growth rate (SGR, % of daily increase in body weight) was similar in WT and GCF fish at all time points measured, except towards harvest size, where WT fish had a higher SGR (0.42 ± 0.13 [females] and 0.45 ± 0.24 [males]) than GCF fish (0.33 ± 0.12 [females] and 0.33 ± 0.20 [males]; $p = 0.0110$ and 0.0002 for WT females vs. GCF females and WT males vs. GCF males, respectively) (Fig. 2A).

Fork length was similar in WT and GCF fish of the same sex at all time points measured. At the terminal sampling in February 2020, WT males

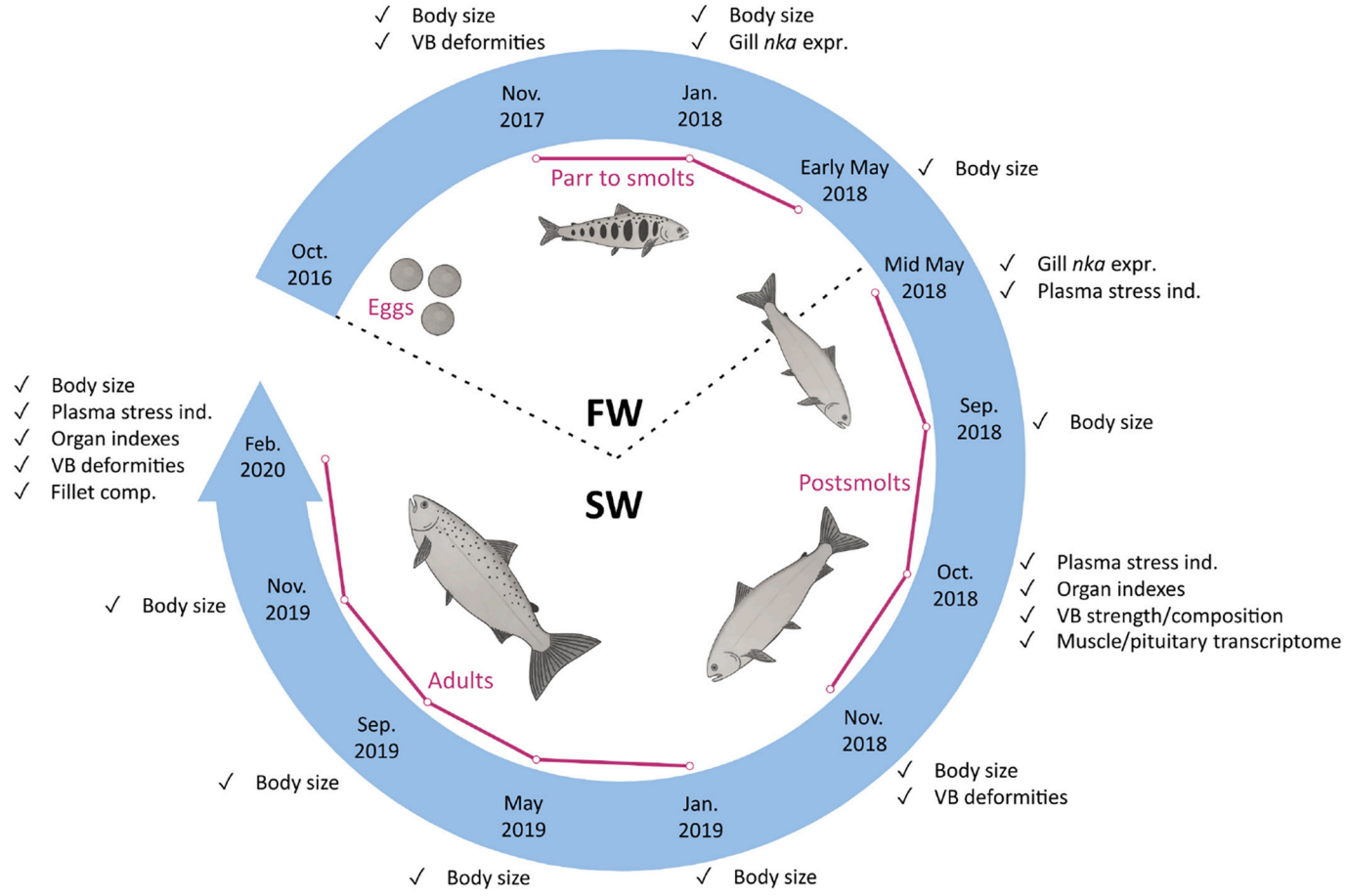

Fig. 1. Overview of the samplings in this study.
Wild type and germ cell-free Atlantic salmon were generated in Oct-Dec. 2016 and followed until Feb. 2020. The twelve sampling points are represented by small circles (and month/year), and the corresponding life stages are indicated. All measurements performed are listed for each sampling. FW, freshwater; SW, seawater; VB, vertebra; expr., expression; ind., indicators; comp., composition.

Table 1
Evaluation of indel rate and lack of germ cells in selected *dnd*-knockout (*dnd*-KO) and wild type (WT) salmon.

Fish ID	Fish group	Gross morph.	Rel. *vasa* expr.	*dnd* indel analysis							
				Gonad				Fin			
				WT	In-frame	Frame-shift	Total reads	WT	In-frame	Frame-shift	Total reads
1	*dnd*-KO	Thin	0.0015	0.39	0.12	99.49	31,538	0.06	0.44	99.50	35,317
3	*dnd*-KO	Thin	0.0012	0.02	1.36	98.63	31,295	0.03	1.53	98.45	30,867
4	*dnd*-KO	Thin	0.0007	0.00	14.72	85.28	22,223	0.03	12.58	87.38	27,472
5	*dnd*-KO	Thin	0.0003	0.00	16.40	83.60	34,402	0.09	23.57	76.33	30,496
9	*dnd*-KO	Thin	0.0018	0.07	0.20	99.73	30,085	0.01	0.19	99.80	32,046
11	*dnd*-KO	Thin	0.0008	0.00	0.06	99.94	28,082	0.07	0.06	99.87	32,272
16	*dnd*-KO	Thin	0.0034	0.79	0.12	99.10	31,681	0.00	0.12	99.88	31,955
18	*dnd*-KO	Thin	0.0005	0.65	2.48	96.88	39,031	0.00	16.60	83.40	39,129
20	*dnd*-KO	Thin	0.0028	1.65	0.20	98.15	21,310	0.00	7.77	92.23	33,366
22	*dnd*-KO	Thin	0.0022	0.11	0.15	99.74	33,073	0.00	0.78	99.22	36,575
24	*dnd*-KO	Thin	0.0004	0.02	0.23	99.76	37,642	0.00	0.26	99.74	40,947
27	*dnd*-KO	Thin	0.0010	0.07	0.29	99.64	38,173	0.03	0.19	99.78	30,352
21	*dnd*-KO	Normal	0.5785	26.72	15.08	58.20	32,714	16.82	2.71	80.48	33,214
12	*dnd*-KO	Normal	1.6733	66.56	0.05	33.39	30,325	30.99	0.12	68.89	40,966
10	WT	Normal	1.7732	99.71	0.00	0.29	28,405	99.71	0.03	0.27	31,597
23	WT	Normal	1.9019	99.80	0.01	0.20	31,779	99.73	0.02	0.25	34,932

Fish ID, fish group, phenotypic observation of gonad at sampling and expression levels of *vasa* (relative to *ef1a*) in gonad tissue is shown. Mutation analysis of gonad and fat fin tissue includes % WT sequence, % in-frame indels, % frameshift indels and total reads.

were longer (78 ± 7 cm) than WT females (76 ± 6; $p = 0.0213$) (Table 2). The daily increase in fork length (mm per day) was similar between all four fish groups up until May 2019. From May to Sept. 2019, WT males had a higher daily increase (1.03 ± 0.20) than WT females (0.92 ± 0.20; $p = 0.0061$). From Sept. to Nov. 2019, both WT and GCF males had a higher length growth (0.74 ± 0.27 and 0.77 ± 0.21, respectively) than WT and GCF females (0.66 ± 0.23 and 0.64 ± 0.10, respectively); $p = 0.0038$ and 0.0005, respectively). From Nov. 2019 to

Table 2
Body size measurements in wild type (WT) and germ cell-free (GCF) Atlantic salmon through a production cycle.

Date	WT females (n = 58–79)	GCF females (n = 28–38)	WT males (n = 51–69)	GCF males (n = 36–56)
Body weight (g)				
Nov. 2017	91 ± 40[a]	73 ± 28	81 ± 38	66 ± 33[b]
Jan. 2018	112 ± 54[a]	88 ± 36	100 ± 50	81 ± 41[b]
May 2018	143 ± 68[a]	115 ± 46	130 ± 65	106 ± 53[b]
Sep. 2018	376 ± 130[a]	314 ± 80	346 ± 97	301 ± 109[b]
Nov. 2018	646 ± 232[a]	550 ± 150	605 ± 187	546 ± 193[b]
Jan. 2019	987 ± 325	880 ± 240	935 ± 277	867 ± 282
May 2019	1845 ± 660	1702 ± 550	1881 ± 534	1688 ± 524
Sep. 2019	3844 ± 1053	3717 ± 792	3938 ± 952	3948 ± 826
Nov. 2019	4394 ± 1063	4141 ± 794	4581 ± 1212	4497 ± 1023
Feb. 2020	5457 ± 1355	4909 ± 915[a]	5828 ± 1684[b]	5382 ± 1462
Fork length (cm)				
Nov. 2017	19 ± 3[a]	18 ± 3	18 ± 3	17 ± 3[b]
Jan. 2018	20 ± 3[a]	19 ± 3	19 ± 3	18 ± 3[b]
May 2018	22 ± 4[a]	21 ± 3	22 ± 4	20 ± 3[b]
Sep. 2018	31 ± 3[a]	30 ± 3	31 ± 3	29 ± 3[b]
Nov. 2018	37 ± 4	36 ± 4	36 ± 4	35 ± 4
Jan. 2019	43 ± 4	42 ± 4	42 ± 4	41 ± 5
May 2019	54 ± 5	52 ± 5	54 ± 4	53 ± 5
Sep. 2019	65 ± 5	66 ± 5	67 ± 5	67 ± 4
Nov. 2019	70 ± 5	70 ± 5	72 ± 6	72 ± 6
Feb. 2020	76 ± 6[a]	75 ± 5[a]	78 ± 7[b]	78 ± 6
Condition factor				
Nov. 2017	1.21 ± 0.05	1.20 ± 0.07	1.20 ± 0.05	1.18 ± 0.06
Jan. 2018	1.24 ± 0.06	1.24 ± 0.06	1.24 ± 0.05	1.23 ± 0.07
May 2018	1.18 ± 0.06	1.18 ± 0.07	1.19 ± 0.05	1.18 ± 0.06
Sep. 2018	1.16 ± 0.09	1.13 ± 0.07	1.17 ± 0.07	1.14 ± 0.07
Nov. 2018	1.25 ± 0.07[a]	1.19 ± 0.09[b]	1.24 ± 0.08[ac]	1.21 ± 0.06[bc]
Jan. 2019	1.23 ± 0.07[a]	1.19 ± 0.07	1.19 ± 0.08[b]	1.19 ± 0.06
May 2019	1.15 ± 0.13	1.14 ± 0.08	1.17 ± 0.12	1.11 ± 0.15
Sep. 2019	1.34 ± 0.14	1.29 ± 0.10	1.28 ± 0.11	1.30 ± 0.10
Nov. 2019	1.25 ± 0.07[a]	1.20 ± 0.10[b]	1.20 ± 0.10[b]	1.20 ± 0.13[b]
Feb. 2020	1.21 ± 0.08[a]	1.14 ± 0.10[bc]	1.17 ± 0.11[ac]	1.12 ± 0.10[b]

Body weight, fork length and condition factor are shown. Data are represented as mean ± SD. Statistical tests, comparing all four groups of fish, included Kruskal-Wallis with Dunn's multiple comparisons test (in cases with no normal distribution), or one-way ANOVA with Tukey's multiple comparisons test (in cases with normal distribution). Significant differences ($p < 0.05$) between fish groups for each parameter and date are indicated by different letters. No letter indicates no significant differences to any other fish group.

Feb. 2020, WT males grew faster in length per day (1.27 ± 0.45) than GCF males (1.15 ± 0.47; $p = 0.0320$) (Fig. 2B).

No difference in condition factor (CF) was detected between any fish group from Nov. 2017 until Sept. 2018 (Table 2). In Nov. 2018, WT females displayed a higher CF (1.25 ± 0.07) than GCF females (1.19 ± 0.09; $p = 0.0014$). In Jan. 2019, CF in WT females was higher (1.23 ± 0.07) than in WT males (1.19 ± 0.08; $p = 0.0403$). In May and Sept. 2019, no differences in CF between any fish group were detected. In Nov. 2019, WT females displayed a higher CF (1.25 ± 0.07) than GCF females (1.20 ± 0.10; $p = 0.0197$). At the terminal sampling in Feb. 2020, WT females had a higher CF (1.21 ± 0.08) than GCF females (1.14 ± 0.10; $p = 0.0043$), and WT males had a higher CF (1.17 ± 0.11) than GCF males (1.12 ± 0.10; $p = 0.0102$).

The change in CF from one sampling to the next (ΔCF) was similar in all fish groups from Nov. 2017 to Jan. 2018, Jan. to May 2018, Sept. to Nov. 2018, Nov. 2018 to Jan. 2019, May to Sept. 2019 and Sept. to Nov. 2019 (Fig. 2C). From May to Sept. 2018, ΔCF in GCF females decreased more (−0.05 ± 0.07) than in WT females (−0.01 ± 0.10; $p = 0.0129$). From Jan. to May 2019, ΔCF in WT females decreased more (−0.07 ± 0.09) than in WT males (−0.02 ± 0.10; $p = 0.0019$). From Nov. 2019 to Feb. 2020, ΔCF decreased more in GCF males (−0.08 ± 0.11) than in WT males (−0.03 ± 0.06; $p = 0.0071$).

3.4. Normal up and downregulation of smoltification markers in gills of GCF salmon transferred from FW to SW

Anadromous Atlantic salmon hatch and spend the first year(s) in FW before migrating to the sea. Before migrating to SW, the fish goes through a developmental process called smoltification or parr-smolt transformation that pre-adapts the fish for the habitat and salinity change associated with SW entry. Importantly, smoltification enables the salmon to develop its hypo-osmoregulatory ability that is required to survive in SW. This ability is associated with increased activity of the enzyme Na^+, K^+-ATPase (NKA) in the gills (Hoar, 1988; McCormick et al., 1998). NKA consists of several subunits. During smoltification, expression of the subunits *α-1b*, *α-3* and *β-1* (SW-type) increase while *α-1a* (FW-type) decreases (Nilsen et al., 2007). In May 2018, all WT and GCF individuals except 3 GCF females and 3 GCF males were phenotypically smolts with silvery body and dark fins. These 6 GCF fish had a parr phenotype and were therefore excluded from all analyses. The 6 GCF parr were reared on in FW and then smoltified one year later in May 2019 (although not included in any analyses). In a random selection of fish (total $n = 16$), we measured mRNA levels of four NKA subunits in gills 4 months before and 24 h after transfer to SW. Repeated gill biopsy over time of the same individual fish showed that the expression of *nka α-1a* decreased from the FW- to the SW-phase in both the WT ($p = 0.0003$) and GCF group ($p = 0.0003$) (Fig. 3A). Conversely, expression of *nka α-1b* (WT $p = 0.0003$, GCF $p = 0.0003$), *nka α-3* (WT $p = 0.0012$, GCF $p = 0.0003$) and *nka β-1* (WT $p = 0.0003$, GCF $p = 0.0003$) increased in both WT and GCF salmon (Fig. 3B, C and D, respectively).

3.5. Transient differences in plasma concentration of biochemical stress markers between GCF and WT salmon

The ability to regulate salt and water balance in body fluids is essential for fish to survive in hypo- and hyper-osmotic environments. This ability can be compromised by different stressors. Changes in plasma osmolality and ion balance are useful markers for acute stress (Sopinka et al., 2016). Another stress indicator is reduction in blood pH due to increased muscle activity and subsequent production of lactic acid. Elevation of the plasma concentration of metabolites such as glucose and lactate may also reflect a stress response in the fish, however care should be taken when interpreting these indicators since they are

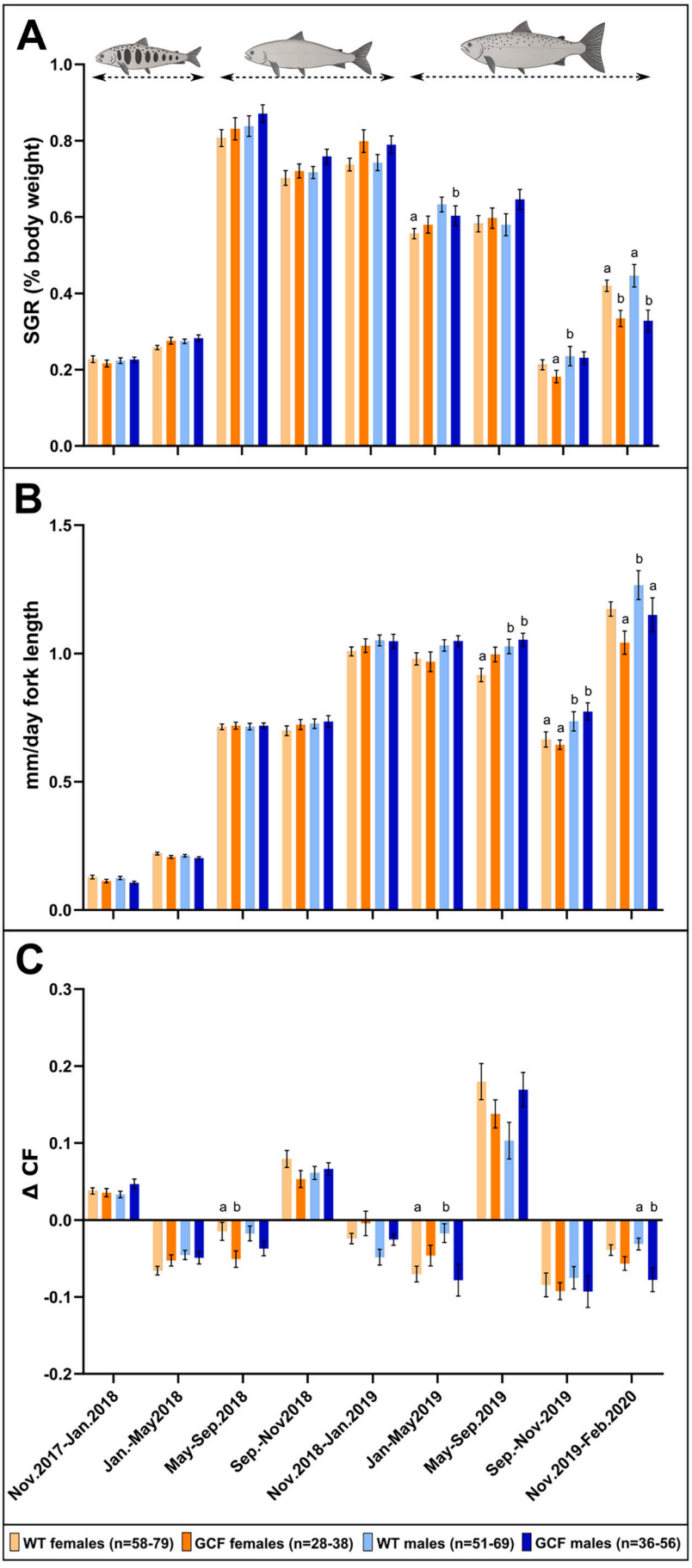

Fig. 2. Growth parameters in wild type (WT) and germ cell-free (GCF) Atlantic salmon, from parr to adults at harvest stage. Specific growth rate (SGR) of body weight (A), daily fork length increase (B) and change in condition factor (CF) (C) are shown. Data are shown as mean ± SEM. Statistical tests, comparing all four groups of fish, included Kruskal-Wallis with Dunn's multiple comparisons test (in cases with no normal distribution), or one-way ANOVA with Tukey's multiple comparisons test (in cases with normal distribution). Significant differences ($p < 0.05$) between fish groups in each sampling are indicated by different letters. No letter indicates no significant differences to any other fish group.

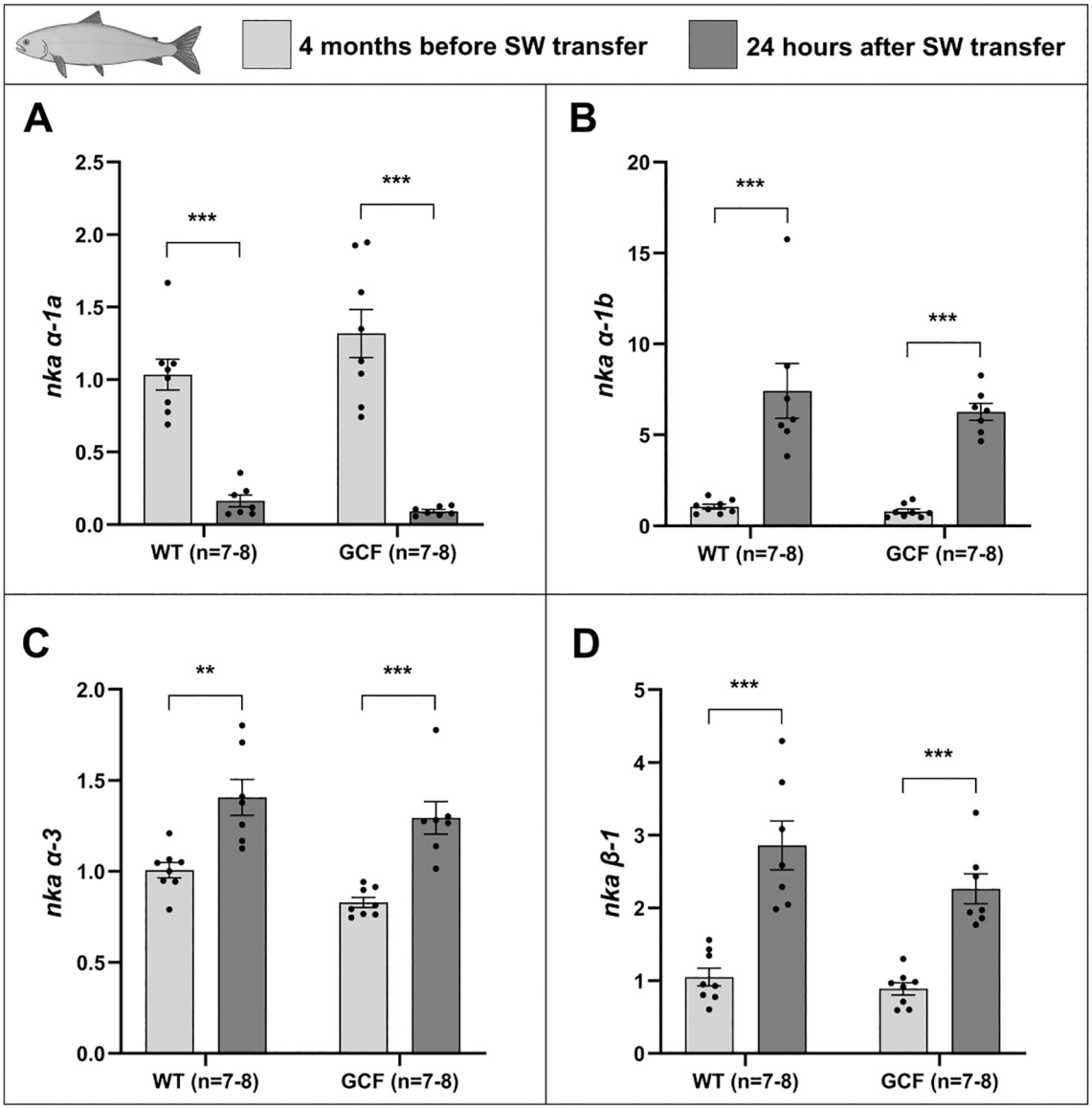

Fig. 3. mRNA levels of Na^+, K^+-ATPase (NKA) subunits in gills of wild type (WT) and germ cell-free (GCF) Atlantic salmon 4 months before and 24 h after transfer to seawater (SW).

Gene expression values for NKA subunits *α-1a* (A), *α-1b* (B), *α-3* (C) and *β-1* (D), are shown relative to *ef1a*. All gene expression values are calibrated to the average of the WT group before SW transfer and are shown as mean ± SEM. A Mann-Whitney test was applied for all statistical comparisons. Significant differences between fish groups are indicated by asterisks (**$p < 0.01$, ***$p < 0.001$).

variable and can be influenced by other non-stress factors (reviewed by Noble et al., 2018). Analysis of plasma concentrations of pH, lactate, sodium (Na^+), chloride (Cl^-) and osmolality in postsmolts after 24 h in SW showed that concentrations of lactate ($p = 0.0217$) and osmolality ($p = 0.0148$) were higher in GCF than WT fish, while no significant differences were detected for plasma pH, Na^+ or Cl^- (Table A.3). In postsmolts after 6 months in SW, and in adults at harvest size, no significant differences were detected in plasma concentrations of pH, glucose, Na^+, Cl^-, calcium (Ca^{2+}) and osmolality when comparing WT and GCF fish of the same sex (Table 3). An additional Mann-Whitney test detected a significant difference in plasma lactate between WT females (2.4 ± 1.0 mmol/L) and GCF females (6.4 ± 3.0; $p = 0.0411$). In adults at harvest size, plasma concentrations of lactate were higher in WT females (9.6 ± 3.5 mmol/L) than in GCF females (7.3 ± 2.8 mmol/L; $p = 0.0087$), and also higher in WT males (8.6 ± 3.7 mmol/L) compared to GCF males (6.9 ± 2.8 mmol/L; $p = 0.0391$). Furthermore, harvest size WT females had lower plasma pH (7.1 ± 0.1) than WT males (7.2 ± 0.1; $p = 0.0137$).

3.6. Normal prevalence of vertebra deformities in GCF salmon

Deformities in the vertebral column may be associated with reduced welfare in salmon (Noble et al., 2018). For instance, an increase in number of deformed vertebrae (DV) is correlated with reduced body growth (Hansen et al., 2010). Certain types of vertebra deformities may also negatively affect swimming performance (Powell et al., 2009). Vertebra deformities are also common causes for downgrading and losses in the salmon farming industry (Michie, 2001). Each individual fish in this study was X-rayed three times during the study period to detect potential differences between WT and GCF salmon in the development of the vertebral column. In Nov. 2017, the occurrence of fish with one or more deformed vertebrae (DV) was 1.4%, 12.5%, 4.7% and 4% for WT females, GCF females, WT males and GCF males, respectively. A year later (Nov. 2018), the prevalence was 5.5%, 18.8%, 6.3% and 12%, while at harvest (Feb. 2020), the proportions were 38.4%, 31.3%, 40.6% and 42% for WT females, GCF females, WT males and GCF males, respectively. Significant difference in the prevalence of DV

between WT and GCF fish could only be detected in postsmolts sampled in 2018 ($p < 0.0498$) (Fig. 4A). The fish that showed one or more DV at any time point were divided into severity categories based on the number of DV; 1–4 DV, 5–8 DV, 9–12 DV, 13–16 DV and > 16 DV (Fig. 4B). Using this classification, in Nov. 2017, no fish in the WT female and GCF male groups had >4 DV, while in GCF females and WT males 27.3% and 3.7% had 5–8 DV. In Nov. 2018, no fish in the WT groups had >5–8 DV, while 9.1% of the GCF females and 4.8% of the GCF males had 9–12 DV. In Feb. 2020, we observed fish within all severity categories up to 16 DV in all four fish groups. Nevertheless, a large proportion (55.2%, 36.4%, 37% and 52.4% for WT females, GCF females, WT males and GCF males, respectively) of the DV individuals were still in the least severe category (1–4 DV). The only group that had any individuals with >16 DV was the WT female group (Fig. 4C). Vertebra stiffness and ash content from vertebra dry weight were also measured in WT and GCF postsmolts after 6 months in SW, however, no differences were detected between any groups (Table A.4).

3.7. Larger relative liver size in WT salmon

Abnormal size of internal organs may be indicative of several conditions, such as disease or suboptimal organ development. Thus, commonly measured organ indexes (HSI and CSI) were compared between WT and GCF postsmolts after 6 months in SW, and adults at harvest size. In postsmolts after 6 months in SW, no significant differences were detected for HSI and CSI when comparing all 4 groups.

Table 3
Biochemical analysis of plasma from wild type (WT) and germ cell-free (GCF) Atlantic salmon postsmolts after 6 months in seawater, and adults at harvest size.

Postsmolts after 6 months in seawater				
Parameter/ group	WT females (n = 6)	GCF females (n = 6)	WT males (n = 5)	GCF males (n = 6)
pH	7.3 ± 0.2	7.3 ± 0.1	7.3 ± 0.1	7.4 ± 0.1
Lactate (mmol/ L)	[2.4 ± 1.0]	[6.4 ± 3.0]	5.2 ± 3.4	4.2 ± 2.8
Glucose (mmol/L)	5.0 ± 0.5	5.4 ± 0.4	5.6 ± 1.2	5.4 ± 1.8
Na^+ (mmol/L)	168 ± 6.5	177 ± 6.6	172 ± 12.8	172 ± 3.6
Cl^- (mmol/L)	138 ± 5.3	142 ± 5.3	140 ± 10.5	140 ± 4.6
Ca^{2+} (mmol/L)	1.31 ± 0.1	1.30 ± 0.1	1.29 ± 0.2	1.28 ± 0.1
Osmolality (Osm/kg)	343 ± 18.5	358 ± 9.9	357 ± 9.8	344 ± 10.9
Adults at harvest size				
Parameter/ group	WT females (*n* = 73)	GCF females (*n* = 32)	WT males (*n* = 64)	GCF males (*n* = 50)
pH	7.1 ± 0.1[a]	7.2 ± 0.1	7.2 ± 0.1[b]	7.2 ± 0.1[b]
Lactate (mmol/ L)	9.6 ± 3.5[a]	7.3 ± 2.8[bc]	8.6 ± 3.7[ac]	6.9 ± 2.8[b]
Glucose (mmol/L)	7.2 ± 2.0	6.7 ± 2.0	6.9 ± 2.3	6.5 ± 1.8
Na^+ (mmol/L)	179 ± 5.0[a]	179 ± 5.0	178 ± 5.7	177 ± 5.4[b]
Cl^- (mmol/L)	145 ± 3.5	147 ± 7.0	145 ± 4.0	145 ± 4.8
Ca^{2+} (mmol/L)	1.31 ± 0.2	1.36 ± 0.2	1.37 ± 0.2	1.34 ± 0.2
Osmolality (Osm/kg)	356 ± 11.4[a]	355 ± 12.7	354 ± 14.0	351 ± 11.2[b]

Plasma pH, lactate, glucose, Na^+, Ca^{2+}, Cl^- and osmolality concentrations are shown. Data are shown as mean ± SD. Statistical tests, comparing all four groups of fish, included Kruskal-Wallis with Dunn's multiple comparisons test (in cases with no normal distribution, or too low n [n < 8] to test for normality), or one-way ANOVA with Tukey's multiple comparisons test (in cases with normal distribution). Significant differences ($p < 0.05$) between fish groups for each parameter are indicated by different letters. No letter indicates no significant difference to any other fish group. Furthermore, an additional Mann-Whitney test was applied for groups with low n ($n \leq 6$), to test for significant differences between WT and GCF fish of the same sex. Significantly different values detected by a Mann-Whitney test are indicated by square brackets.

However, an additional Mann-Whitney test detected higher HSI in WT females (1.4 ± 0.08) compared to GCF females (1.27 ± 0.08; $p = 0.0152$) (Fig. 5A). At harvest size, HSI was higher in the two WT groups (1.07 ± 0.21/females and 1.01 ± 0.12/males) compared to the GCF groups (0.91 ± 0.09/females and 0.92 ± 0.12/males; $p < 0.0001$ for WT females vs. GCF females, $p = 0.0018$ for WT males vs. GCF males). CSI was higher in WT males (0.15 ± 0.03) than WT females (0.13 ± 0.02, $p = 0.0414$) (Fig. 5B).

3.8. No sexual maturation in GCF salmon

Relative gonad size and plasma concentration of sex steroids were compared between WT and GCF salmon to search for potential differences in sexual maturation. Postsmolt WT females displayed higher GSI (0.13 ± 0.04) than GCF females (0.06 ± 0.03, $p = 0.0439$), while no difference was detected between WT and GCF males (Fig. 6A). At harvest size, all fish groups had different GSI; WT females had the highest value (0.67 ± 0.33) followed by WT males (0.28 ± 0.24), GCF males (0.04 ± 0.02) and finally GCF females (0.01 ± 0.00; $p < 0.0001$ for all comparisons) (Fig. 6B). At harvest size, we visually observed a number of sexually mature (ovulated females and running males) WT individuals. One WT female (1.4%) and 7 WT males (10.9%) were sexually mature, while no GCF fish showed any visual signs of maturation (secondary sex characteristics). In a random selection of 15 WT and 15 GCF females, plasma E_2 was undetectable in all GCF individuals, while WT females had an average concentration of 0.35 ± 0.32 ng/mL ($p < 0.0001$). Likewise, in a random selection of 15 WT and 15 GCF males, WT individuals displayed higher plasma 11-KT (4.24 ± 2.92 ng/mL) and T (1.04 ± 0.84 ng/mL) than GCF males (0.95 ± 1.50 and 0.10 ± 0.21 ng/mL, respectively; $p = 0.0203$ and 0.0006, respectively) (Fig. 6C).

3.9. Higher relative proportion of EPA and DHA in fillets from GCF salmon males

Fatty acid composition was compared between randomly selected WT and GCF salmon adults at harvest size (total $n = 22$), to investigate possible differences in fatty acid fillet quality. When comparing all four groups of fish, no significant differences were found on growth parameters (body weight, fork length, condition factor) or proximate composition of protein (g/100 g wet weight [ww]), dry matter (g/100 g) or total fat (g/100 g ww), although WT salmon had slightly higher (but not significant) level of total fat (12.6 ± 6.1 [females], 13.2 ± 3.3 g/100 g [males]) compared to GCF salmon (10.8 ± 3.6 [females], 9.2 ± 3.0 g/100 g [males]). However, when comparing only WT with GCF fish of the same sex, the dry matter % (g/100 g) was significantly higher in WT males (35.3 ± 0.5) than in GCF males (33.6 ± 1.4; $p = 0.0177$) (Table A.5).

Fillet fatty acid composition (mg/g ww) of 18:1n-9, 18:2n-6 and 18:3n-3 was lower in GCF males (35.3 ± 5.2, 11.5 ± 1.6 and 3.9 ± 0.6) than in WT males (47.9 ± 9.8, 15.4 ± 2.8, 5.3 ± 0.9; $p = 0.0318$, 0.0419, 0.0469, respectively) (Table A.5). An additional test comparing only WT with GCF fish of the same sex, also revealed lower 14:0, 16:0, sum of monounsaturated fatty acids (MUFA) and sum of n-6 in GCF males (3.5 ± 0.7, 13.5 ± 2.3, 53.0 ± 8.0, 14.0 ± 2.0) compared to WT males (4.5 ± 0.9, 17.8 ± 3.9; $p = 0.0480$, 0.0480, 0.0177, 0.0177, respectively). Furthermore, the same test detected a significantly higher n-3/n-6 ratio in GCF males (1.7 ± 0.1) compared to WT males (1.6 ± 0.1; $p = 0.0139$). When the data were normalized to total lipid content (% of total fatty acids), most of these differences disappeared (Table 4). However, the relative content of the long chain polyunsaturated fatty acids EPA (docosapentaenoic acid/20:5n-3) and DHA (docosahexanoic acid/22:6n-3) was higher in GCF males (4.0 ± 0.1, 8.4 ± 0.3%) compared to WT males (3.7 ± 0.1, 7.5 ± 0.3%; $p = 0.0063$, 0.0037) despite having the lowest total amount (mg/g) of fatty acids (Table 4).

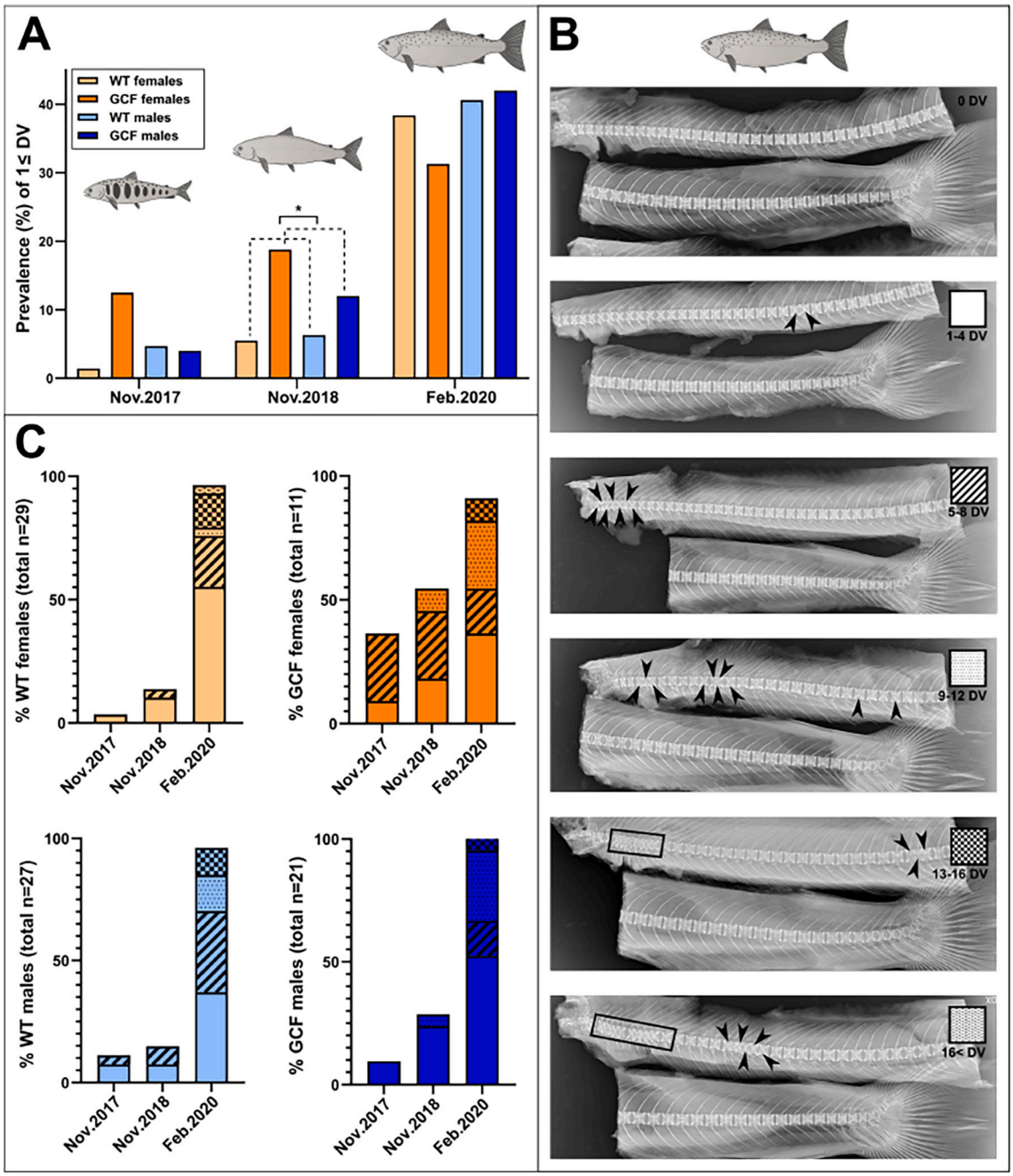

Fig. 4. Prevalence of deformed vertebra (DV) in wild type (WT) and germ cell-free (GCF) Atlantic salmon.
A) Proportion of salmon with 1 ≤ DV in Nov. 2017 (parr), Nov. 2018 (postsmolts) and Feb. 2020 (adults at harvest size). Total n (100%) = 73 (WT females), 32 (GCF females), 64 (WT males), 50 (GCF males). B) Vertebra deformity categories applied in this study based on number of DV; 0, 1–4, 5–8, 9–12, 13–16 and 16 < DV. DV are indicated by black arrows or squares. C) DV severity (according to the categories in B) in individuals with DV at one or more time points; proportion of WT females (upper left), GCF females (upper right), WT males (lower left) and GCF males (lower right) in the different severity groups over time is shown. A Fisher's exact test was applied to test for significant differences between WT and GCF fish (mixed sex). Significant differences between fish groups are indicated by asterisks (*p < 0.05).

3.10. Miniscule differences between WT and GCF pituitary and muscle transcriptomes

When knocking out a gene exclusively expressed in germ cells, which results in the loss of these cells, the expected initial molecular and morphological effects are confined to gonads, which in turn affect endocrine pathways associated with germ cell signaling to the surrounding cells and other tissues connected via the brain-pituitary-gonad axis. Indeed, direct endocrine effects of knocking out *dnd* (and subsequent loss of all germ cells) in salmon has been documented previously (Wargelius et al., 2016), and effects on sex steroid production (Kleppe et al., 2017) and transcriptomic changes in testis tissue have been shown (Kleppe et al., 2020). However, it is unknown if there may be other systemic effects elsewhere in the fish body. To explore this at the molecular level, we compared the transcriptomes of selected tissues in WT and GCF postsmolts after 6 months in SW. The results may reflect systemic physiologic effects (pituitary) or changes in the edible tissue (muscle) in response to the *dnd* mutation and subsequent lack of germ cells. In total, only 11 differentially expressed genes (DEGs) were identified (Table A.6). 5 DEGs (upregulated in the GCF group) were

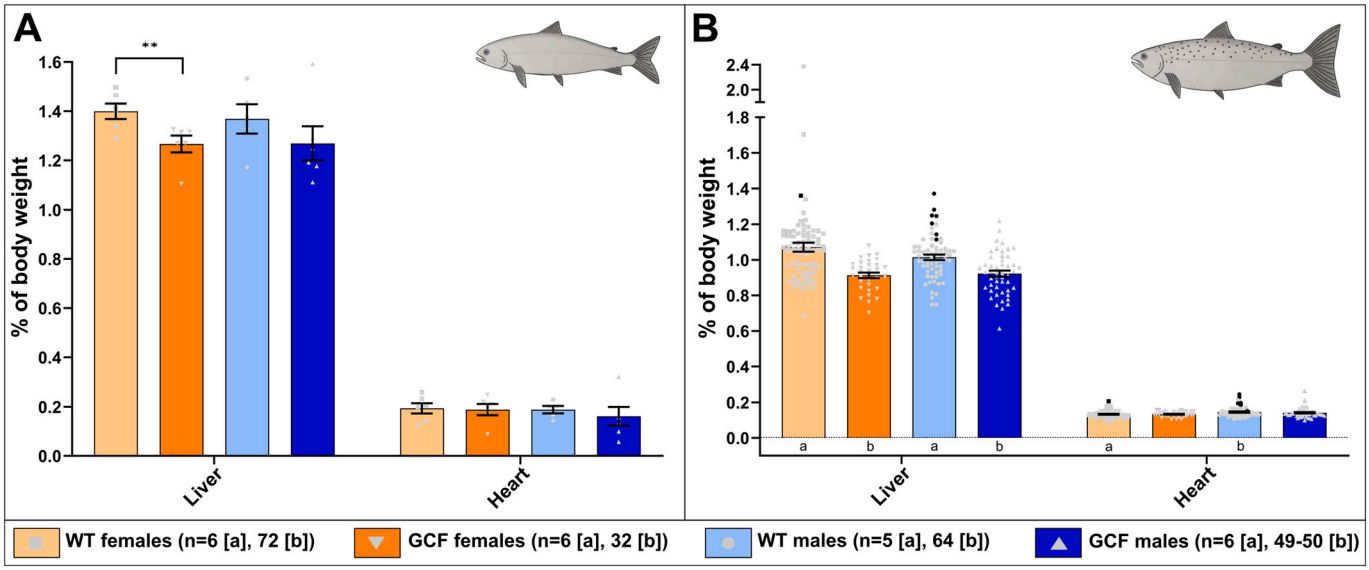

Fig. 5. Relative organ size in wild type (WT) and germ cell-free (GCF) Atlantic salmon postsmolts after 6 months in seawater (A) and adults at harvest size (B). Relative size (% of body weight) of liver and heart is shown as mean ± SEM. Values from sexually mature (ovulating [females] or running [males]) individuals are given as black dots. Statistical tests, comparing all four groups of fish, included Kruskal-Wallis with Dunn's multiple comparisons test. Significant differences ($p < 0.05$) between fish groups are indicated by different letters. No letter indicates no significant differences to any other fish group. Furthermore, an additional Mann-Whitney test was applied for groups with low n ($n \leq 6$; A), to test for significant differences between WT and GCF fish of the same sex. Significantly different values detected by a Mann-Whitney test are indicated by asterisks (**$p < 0.01$).

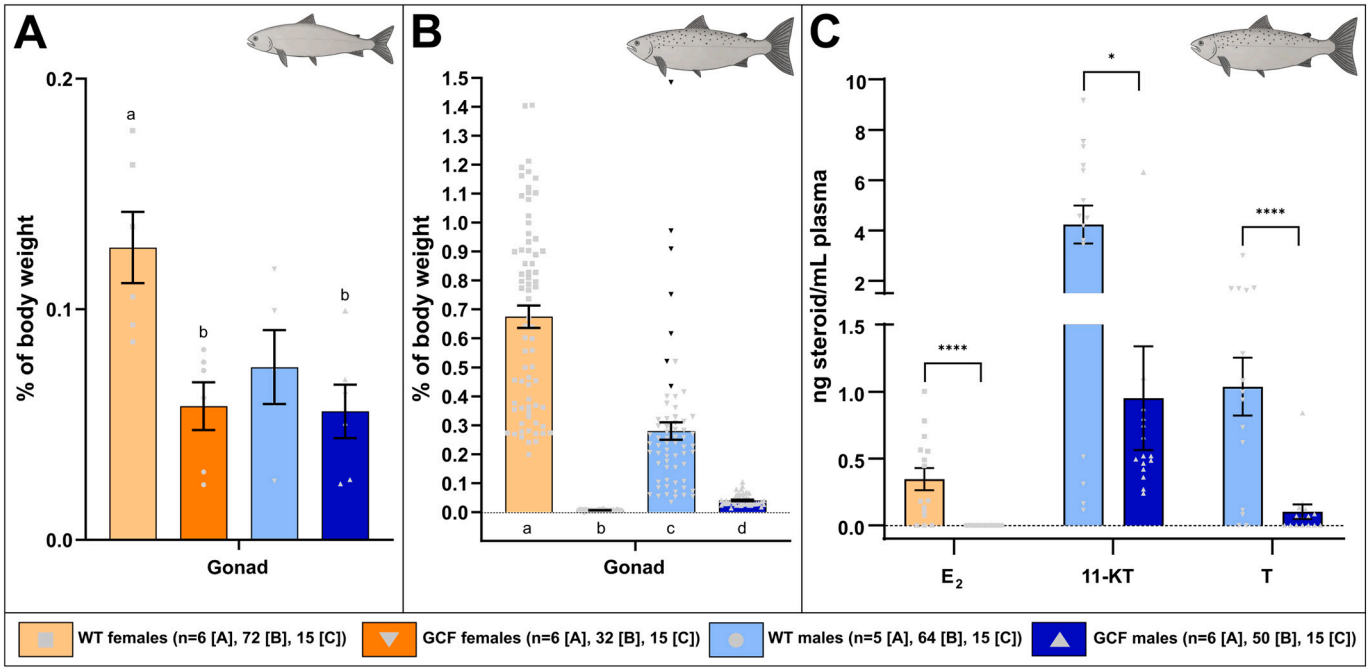

Fig. 6. Relative gonad size in wild type (WT) and germ cell-free (GCF) Atlantic salmon postsmolts after 6 months in SW (A) and adults at harvest size (B), and plasma concentrations of sex steroids in adults at harvest size (C).
Relative size (% of body weight) of gonads is shown as mean ± SEM. Values from sexually mature (running) males are given as black dots. Statistical tests, comparing all four groups of fish, included Kruskal-Wallis with Dunn's multiple comparisons test. Significant differences ($p < 0.05$) between fish groups are indicated by different letters. No letter indicates no significant differences to any other fish group (A, B). Furthermore, an additional Mann-Whitney test was applied for groups with low n ($n \leq 6$), to test for significant differences between WT and GCF fish of the same sex. However, no additional differences were detected by the Mann-Whitney test (A). Plasma concentrations (ng/mL) of estradiol-17β (E2) in randomly selected immature-looking females, and 11-ketotestosterone (11-KT) and testosterone (T) in randomly selected immature-looking males are shown as mean ± SEM. A Mann-Whitney test was applied for all statistical comparisons. Significant differences between fish groups are indicated by asterisks (*$p < 0.05$, ****$p < 0.0001$) (C).

Table 4
Relative fatty acid composition (% of total fatty acids) in wild type (WT) and germ cell-free (GCF) Atlantic salmon fillets.

Relative fatty acid composition (% of total fatty acids)				
Parameter	WT females (n = 5)	GCF females (n = 5)	WT males (n = 5)	GCF males (n = 7)
14:0	3.1 ± 0.1	3.0 ± 0.1	3.0 ± 0.1	3.0 ± 0.2
16:0	12.1 ± 0.4	11.9 ± 0.2	11.7 ± 0.2	11.6 ± 0.4
18:0	3.0 ± 0.1	3.0 ± 0.2	3.0 ± 0.2	3.0 ± 0.1
Sum SFA	19.1 ± 0.7	18.8 ± 0.4	18.6 ± 0.3	18.5 ± 0.6
18:1n-9	30.3 ± 0.8	30.9 ± 1.1	[31.6 ± 0.4]	[30.5 ± 0.7]
Sum MUFA	45.8 ± 0.8	46.1 ± 0.9	[46.7 ± 0.3]	[45.8 ± 0.5]
18:2n-6	9.6 ± 0.4	10.1 ± 0.4	10.2 ± 0.3	10.0 ± 0.5
Sum n-6	11.7 ± 0.3	12.1 ± 0.4	12.3 ± 0.2	12.1 ± 0.4
18:3n-3	3.3 ± 0.1	3.4 ± 0.1	3.5 ± 0.1	3.4 ± 0.2
EPA 20:5n-3	4.0 ± 0.2	4.0 ± 0.3	[3.7 ± 0.1]	[4.0 ± 0.1]
DHA 22:6n-3	7.8 ± 0.4	7.9 ± 0.3	[7.5 ± 0.3[a]]	[8.4 ± 0.3[b]]
EPA + DHA	11.8 ± 0.5	11.9 ± 0.6	[11.3 ± 0.3[a]]	[12.4 ± 0.4[b]]
n-3/n-6 ratio	1.7 ± 0.1	1.7 ± 0.1	[1.6 ± 0.1]	[1.7 ± 0.1]
Sum fatty acids (mg/g)	150.6 ± 22.3[a]	140.4 ± 20.4	[151.6 ± 30.8]	[115.8 ± 17.9[b]]

Data are shown as mean ± SD. Statistical tests, comparing all four groups of fish, included Kruskal-Wallis with Dunn's multiple comparisons test. Significant differences ($p < 0.05$) between fish groups for each parameter are indicated by different letters. No letter indicates no significant difference to any other fish group. Furthermore, an additional Mann-Whitney test was applied to test for significant differences between WT and GCF fish of the same sex. Significantly different values detected by a Mann-Whitney test are indicated by square brackets. SFA; saturated fatty acids, MUFA, monounsaturated fatty acids, PUFA; polyunsaturated fatty acids.

identified when comparing WT with GCF female pituitaries: two *von Willebrand factor A domain-containing protein 7-like* (*vwa7l*), *CAP-Gly domain-containing linker protein 1-like* (*clip1l*), *peptidyl-prolyl cis-trans isomerase FKBP5-like* (*fkbp5l*) and *ChaC, cation transport regulator-like 1* (*chac1*). When comparing WT with GCF male pituitaries, 4 genes were downregulated in the GCF group: *clusterin* (*clu*), *somatotropin-2* (*gh2*), *V-set and transmembrane domain-containing protein 2-like* (*vstm2l*) and *glycoprotein hormone beta 5* (*gphb5*). Finally, we identified 2 DEGs when comparing WT with GCF female muscle tissue: *serine/threonine-protein kinase pim-1-like* (*pim1l*; upregulated in the GCF group) and an unknown gene (downregulated in the GCF group). No DEGs were identified when comparing muscle tissue from WT and GCF males (Table A.6).

4. Discussion

We have compared the long-term performance of WT and *dnd* mutant GCF salmon. When working with mutation-induced phenotypes in the F0 generation there is always a risk of obtaining mosaic phenotypes, as we previously have observed in Atlantic salmon (Wargelius et al., 2016). We ensured to analyse exclusively GCF individuals by verifying that all fish included in the evaluations had 1) thin gonads classified as apparently "empty", and 2) very low/undetectable gonadal expression of *vasa*. In addition to *dnd*, we also induced a mutation in the *slc45a2* gene in the same individuals, since the resulting phenotype of lacking pigmentation served as a visual tracer for successful knockout of the target gene *dnd* (Wargelius et al., 2016). Although we do not expect any other effects than the lack of pigment from the *slc45a2* mutation, we cannot completely rule this out.

No significant differences were detected when comparing body size of WT and GCF salmon. However, a trend towards larger WT fish towards harvest size, in combination with significantly higher CF and growth rates in both WT females and males compared to their GCF counterparts, suggests that WT fish had a growth advantage during this period of their life cycle. Limited information exists about the effect of germ cells on body growth. In a previous study, we found no clear effect on growth from the lack of germ cells in postsmolts exposed to a maturation-inducing rearing regime (Kleppe et al., 2017). Furthermore, a study on *dnd*-knockdown zebrafish reported similar body weight in GCF males and immature control males at three months age (Wong and Zohar, 2015b). On the other hand, gonads have a significant growth promoting effect in female and male Mozambique tilapia, related to gonadal growth hormone release and other mechanisms and factors, including sex steroids (Bhatta et al., 2012). Indeed, our GCF salmon model showed lower levels of sex steroids than their maturing WT counterparts. While body growth is a complex process, it seems likely that low or undetectable levels of sex steroids may have dampened growth of GCF salmon (Youngson et al., 1988). Given the observed differences in growth rate, and the well-known decrease in growth in maturing salmon after the initial, puberty-associated growth spurt (Taranger et al., 2010), we suggest that it would be advantageous to harvest GCF salmon later in the year than their WT counterparts, allowing GCF salmon to grow to a larger size than WT individuals that experience increasing growth inhibition due to progressing sexual maturation. CF in fish changes with season and life stage. However, a fish with CF < 0.9 is considered to have reduced welfare (Stien et al., 2013). The average CF of WT and GCF salmon was similar throughout a large extent of this study and always higher than 1.1, well within values considered as healthy. Taken together, while WT fish trended towards becoming bigger than their GCF counterparts towards harvest size, they had similar growth rates throughout most of the production cycle, and they were always within a healthy CF range.

Analysis of smoltification markers in the gills of WT and GCF salmon prior to, and after SW transfer, showed significant upregulation of the SW-type subunits and significant downregulation of the FW-type in both groups, indicating a normal smoltification capacity in GCF fish. However, a random selection of GCF fish that had just been transferred to SW showed a higher osmolality and lactate concentration than their WT counterparts, which may point to a higher sensitivity to stressors or a more demanding smoltification process in GCF smolts. Later in the life cycle, only the lactate plasma concentration differed when comparing WT with GCF fish. Since lactate was first (after 6 months in SW) highest in GCF fish (females), but then shifted and became highest in WT fish at harvest size, there is no clear general trend towards higher stress in either of the fish groups. Taken together, there is no clear indication that the lack of germ cells is causing a significant imbalance in the aspects of body fluid homeostasis examined here, and if any it appears to be transient.

DV were observed in all fish groups at all time points studied, with increasing prevalence and severity with time. Although a higher % of GCF fish had one or more DV at one time point, this difference disappeared later (prevalence ranging from 31.3 to 42%). All severity categories up to 16 DV were observed in all fish groups at the final sampling. Previous studies have reported DV prevalences (% fish with 1 or more DV) ranging from 12 to 94% (Witten et al., 2006; Fjelldal et al., 2007; Fjelldal et al., 2009; Hansen et al., 2010; Grini et al., 2011) in cultured adult salmon. Furthermore, adult wild Atlantic salmon displayed prevalences of 5, 19, 29 (Sambraus et al., 2014) and 35% (Fraser et al., 2014), showing that even wild salmon may have up to the same prevalence as has been observed in farmed salmon. The prevalence of DV observed in this study, for all fish groups, are within normal occurring prevalence in Atlantic salmon; we therefore demonstrate that GCF salmon are not more prone to develop DV than their WT counterparts. In the case of triploid sterile salmon, a higher amount of dietary phosphorous can prevent vertebra deformities (Fjelldal et al., 2016). However, phosphorous is a limited resource, and increased use of phosphorous causes increased pollution and eutrophication. Since GCF salmon develop normally with a standard diet (which is also supported by similar vertebra stiffness and mineral content in WT and GCF salmon after 6 months in SW [Table A.4]), less phosphorous would be required to farm GCF salmon. Hence, the GCF sterility model may be a more sustainable choice than the current use of triploid Atlantic salmon.

Regarding internal organ size, no difference in CSI was detected between WT and GCF salmon, suggesting that GCF salmon had normal heart development. HSI was similar in WT and GCF fish after 6 months in SW, but there was a tendency (detected with one out of 2 statistical tests) towards larger relative liver size in WT females compared to GCF females. This trend continued, resulting in significantly higher HSI in WT compared to CGF females and males at harvest size. These results may be explained by pubertal processes taking place in WT fish (see below). The liver is an important organ for female and male reproduction in fish. In females, during oogenesis, E_2 from the ovary stimulates hepatocytes of the liver to produce vitellogenin, the main precursor of yolk proteins that accumulate in the growing oocytes during vitellogenesis (Tyler and Sumpter, 1996; Senthilkumaran et al., 2004). It is known that E_2-induced vitellogenin synthesis in salmonid livers causes an increase in liver weight, and changes in morphology and RNA and DNA content (Olin and von der Decken, 1987; Mackay and Lazier, 1993). Studies in diploid and triploid female salmonids also found an increase in HSI in response to E_2-treatment (Benfey et al., 1989; Krisfalusi and Cloud, 1996). In contrast, another study (Schafhauser-Smith and Benfey, 2003) found no effect on HSI following long-term E_2-treatment of female triploid brook trout. The authors suggested that a possible effect on liver size may have been counteracted by the long-term E_2-treatment. In male teleosts, 11-KT is considered as the major androgen involved in spermatogenesis (Borg, 1994). To our knowledge, no studies have investigated effects of 11-KT on HSI in salmon, however, 11-KT treatment was found to increase liver size in short-finned female eels (Rohr et al., 2001). In Atlantic cod it has been shown that HSI changes during the reproductive cycle, with lower HSI as well as GSI and sex steroids just after spawning in both sexes (Dahle et al., 2003). In humans, injections of T increased the liver volume in a dose-dependent manner (Gagliano-Jucá et al., 2017). Similarly, administration of androgens increased liver size in rats (Ferrández et al., 1996; Friedel et al., 2006; Hickson et al., 1976; Vieira et al., 2008). Hence, we speculate that the higher concentration of 11-KT or T (or both), in addition to the higher growth rate in WT compared to GCF males towards the end of the experiment, may have stimulated liver growth in WT males.

Based on visual inspection of the gonads at the terminal harvest sampling, a small proportion of WT females (1.4%) and males (10.9%) were classified as fully mature (ovulating or running). On the contrary, no mature GCF individuals were observed, which is in agreement with our previous findings on GCF postsmolts reared under maturation-inducing conditions (Kleppe et al., 2017). In the current study, GCF salmon had very low GSI values and plasma sex steroid levels. In contrast to GCF males (that had <1 ng/mL 11-KT in plasma), most of the "immature-looking" WT males had concentrations higher than 3 ng/mL, suggesting that puberty had been initiated, which is also supported by their GSI values being higher than 0.1% (Kjærner-Semb et al., 2018; Fraser et al., 2019). A few WT males showed similar 11-KT plasma concentration as GCF males, however, the GSI values in most of those WT individuals were still $\geq$0.1%, in contrast to the GCF males (Table A.7). Based on plasma E_2 (0.35 ± 0.32 ng/mL), the time of the year (February) and GSI (0.67 ± 0.33), WT females had potentially started the process of becoming prepared to mature the following autumn (Andersson et al., 2013), in strong contrast to the GCF females that showed clearly lower E_2 levels and GSI values. These findings suggest that GCF salmon females and males, in contrast to their WT counterparts, never initiate sexual maturation, which is a clear advantageous trait with respect to the concern of genetic introgression of farmed escapees with wild populations, as well as maturation-related welfare challenges. Furthermore, the lack of maturation in GCF salmon opens the opportunity to let them grow to a larger size without risking maturation-associated welfare problems. Finally, since maturing salmon may show reduced production performance parameters such as fillet quality (Taranger et al., 2010), which is one of the causes for downgrading of the end product in the salmon industry (Michie, 2001), using GCF salmon could eliminate such challenges. In comparison to sterile triploid salmon, the GCF model (males) may be a more sustainable choice for farmers since male triploids mature despite being sterile. Furthermore, if mature triploid males escape, they can interfere with the spawning of wild salmon by attempting to spawn with wild females (Fjelldal et al., 2014).

Despite receiving the same quantity, quality and composition of feed over the same period of time, there were significant differences in fillet fatty acid composition between WT and GCF salmon males. Our results revealed that quantities (mg/g ww) of some fatty acids were lower in GCF males than in WT males. However, the amount of essential polyunsaturated fatty acids including EPA and DHA did not differ significantly between any fish group, suggesting that GCF salmon can match the healthy fatty acid content (amount of omega-3 per unit of fillet) of their WT counterparts. The higher amount of other fatty acids in WT males compared to GCF males was reflected in a lower relative amount of EPA and DPA in WT males, when normalizing the fatty acid composition to the total lipid content. The same pattern was reported when comparing reproductive function and fatty acid fillet quality between triploid and diploid farmed Atlantic salmon. The authors found that triploids were leaner than diploids, but the relative levels of important omega-3 long chain polyunsaturated fatty acids were higher in triploids (Murray et al., 2018). Although the genetic basis of Atlantic salmon produced by the novel CRISPR/Cas9 technology and triploidization are very different, both techniques target the same phenotypic outcome namely sterility. Our results, together with the findings by Murray et al., 2018, suggest that sterile Atlantic salmon may have an altered composition of fatty acids, with a higher (relative) proportion of omega-3 fatty acids. The fact that we only observed significantly higher relative levels of EPA, and in particular DHA, in male GCF individuals compared to male WTs, could be related to their potential role(s) in male sexual maturation. A recent study highlighted the importance of DHA in sexually maturing salmon males compared to immature fish, reporting increased testis expression of genes involved in elongation/desaturation processes which produces DHA, in the sexually maturing group (Bogevik et al., 2020). Since we observed signs of pubertal development (discussed above) in WT but not in GCF males at harvest size, DHA may have been redistributed from somatic tissue to testis tissue, thereby lowering the relative levels of DHA in the fillet of WT salmon. Higher relative levels of DHA in GCF males could also be associated with a general reduced requirement into costly reproductive maturation (Cleveland et al., 2012). However, further research is required to elucidate the genetic explanation for the observed fillet fatty acid differences between GCF and WT male salmon.

When comparing the pituitary and muscle transcriptomes in WT and GCF postsmolt females and males after 6 months in SW, none or very few DEGs were detected. This suggests none or very limited effects from the loss of *dnd* function or the lack of germ cells (or both) in these extragonadal tissues at that life stage. However, interestingly, one of the DEGs detected when comparing WT male with GCF male pituitaries is *gh2*, which is one of two paralogs of *growth hormone* in Atlantic salmon (von Schalburg et al., 2008). Growth Hormone (Gh) is the principal regulator of skeletal growth in salmonids, but is also involved in other complex processes such as seawater adaptation and sexual maturation (Björnsson, 1997). Although there were very few differences in any performance parameter measured in WT and GCF postsmolts after 6 months in SW, we did observe that differences developed later, both in terms of body growth rates, CF and sexual maturation. An increased expression of *gh2* in WT postsmolt pituitaries could potentially precede differences observed later. In case of comparing the pituitary and muscle transcriptomes at a later life stage, when the WT (but not the GCF) fish would be going through and complete puberty, we would likely detect clear differences since the pituitary is an essential part of the brain-pituitary-gonad axis controlling puberty (Zohar et al., 2010), and also since we detected differences in the fatty acid composition in fillets. While such differences would be expected, future studies will have to clarify if any effects may be observed in additional tissues and life stages.

5. Conclusions

We have described the long-term performance (one production cycle), of *dnd*-KO, GCF and thus sterile, Atlantic salmon. GCF salmon maintained a similar body size as their WT counterparts throughout the study period. However, there was a trend towards a growth advantage in the WT group as they were reaching harvest size, reflected by their higher CF and growth rates, in line with signs of puberty initiation. Regarding GCF salmon, that we observed to never become sexually mature, it might be beneficial to postpone the time of harvest to exploit the growth potential uninhibited by sexual maturation, to obtain larger fillets. Molecular markers suggested that both WT and GCF fish had normal smoltification capacity. Moreover, we found no clear indication that GCF salmon were more stressed than WT individuals. Very small differences were detected between the transcriptomes of pituitary and muscle tissues in GCF and WT postsmolts. GCF salmon were not more prone to develop vertebra deformities than their WT counterparts. Relative heart size measurements indicated normal development of the heart in GCF fish, while a larger liver in the WT group towards harvest size may reflect reproductive-associated processes. Importantly, GCF salmon were able to match the healthy fatty acid content of their WT counterparts, and our results indicate an altered fatty acid composition in GCF males with higher relative proportion of essential omega-3 fatty acids than WT males. Regarding sexual maturation, GCF salmon (in contrast to WTs) have the advantage of never maturing, which eliminates the risk of developing maturation-related welfare issues. Compared to sterile triploid salmon currently used in the industry, the GCF sterility model may be a more sustainable choice since triploid males are able to go through sexual maturation. Furthermore, GCF salmon would potentially have less problems with downgrading losses at slaughter than triploids since triploids are more prone to develop skeletal deformities. Based on our findings, the GCF salmon is a promising sterility model for potential commercial use in the future. However, more studies are required to evaluate how this model performs in commercial production systems. For instance, no information exists on how GCF salmon cope in sea cages. It is also highly relevant to test the performance of GCF salmon in recirculating aquaculture systems, where currently precocious maturation is a significant challenge with respect welfare and growth. Additional welfare aspects should be monitored, both physical indicators from the fish environment and from a behavioral perspective. Furthermore, knowledge about markets and consumer acceptance will be important for the evaluation of the potential use of GCF salmon in aquaculture.

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Data availability

The raw RNA sequencing data are available at https://www.ncbi.nlm.nih.gov/bioproject/PRJNA764865.

CRediT authorship contribution statement

L. Kleppe: Conceptualization, Methodology, Formal analysis, Investigation, Visualization, Writing – original draft. **P.G. Fjelldal:** Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft. **E. Andersson:** Conceptualization, Methodology, Investigation, Writing – review & editing. **T. Hansen:** Conceptualization, Methodology, Investigation, Writing – review & editing. **M. Sanden:** Conceptualization, Methodology, Formal analysis, Writing – original draft. **A. Bruvik:** Investigation. **K.O. Skaftnesmo:** Investigation. **T. Furmanek:** Formal analysis, Data curation, Writing – review & editing. **E. Kjærner-Semb:** Formal analysis, Writing – review & editing. **D. Crespo:** Investigation, Writing – review & editing. **S. Flavell:** Investigation, Writing – review & editing. **A.Ø. Pedersen:** Investigation. **P. Vogelsang:** Investigation, Writing – review & editing. **A. Torsvik:** Investigation. **K.A. Kvestad:** Investigation, Writing – review & editing. **S. Olausson:** Investigation. **B. Norberg:** Formal analysis, Writing – review & editing. **R.W. Schulz:** Conceptualization, Methodology, Writing – review & editing. **J. Bogerd:** Conceptualization, Methodology. **N. Santi:** Conceptualization, Resources. **R.B. Edvardsen:** Conceptualization, Methodology, Writing – review & editing. **A. Wargelius:** Conceptualization, Methodology, Funding acquisition, Investigation, Writing – original draft.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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