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1 **Production of sterile Atlantic salmon by germ cell ablation with antisense**
2 **oligonucleotides**

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14 Keywords: sterile salmon, Morpholino, Gapmer, antisense oligonucleotides,
15

16 **Abstract**

17 Cultivation of sterile-only fish in aquaculture offers multiple benefits of environmental,
18 economical, and social value. A reliable method for efficient sterilization without
19 affecting fish welfare and performance traits would have significant impact on fish
20 production practices. Here, we demonstrate sterilization of Atlantic salmon embryos by
21 targeting the *dead end* gene with antisense oligonucleotides. Successful gene knock
22 down and sterilization was achieved only when using Gapmer oligonucleotides and not
23 with morpholino oligos. Germ cell-depleted embryos developed into morphologically
24 normal male and female salmon with rudimentary gonads devoid of gametes.
25

26 **Introduction**

27 Atlantic salmon (*Salmo salar*) is one of the most important aquaculture species with a
28 total worldwide production exceeding 2.74 million tons in 2021(M. Shahbandeh, 2020).
29 The species is also ecologically and culturally important, and an iconic target in
30 recreational fishing. Intensive Atlantic salmon production mostly takes place in sea
31 cages. Despite constantly improving quality of the equipment and implementation of
32 stricter requirements for biosecurity, the number of escaping farmed fish is raising
33 environmental issues for the genetic integrity of the wild stocks(Bolstad et al., 2021).
34 In aquaculture production, salmon are commonly cultured over 3 years and harvested
35 before reaching sexual maturity involving dramatic changes in physiology, behavior,
36 and morphology(Taranger et al., 2010). Although beneficial to the species in their

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37 natural environments, the variability in maturation timing is a significant problem for the
38 salmon farmers. Specifically, early maturing fish often exhibit decreased growth and
39 feed conversion efficiency(Mobley et al., 2021), reduced product quality(Davidson et
40 al., 2018), and increased susceptibility to opportunistic microorganisms(Oidtmann et
41 al., 2013), all causing economic loss(Rivera et al., 2022). Over the years, in an attempt
42 to reduce precocious maturation, salmon farming industry has adopted various
43 strategies such as photoperiod control(Bromage et al., 2001) and selective breeding
44 for late maturation(Iversen et al., 2016) with varying success overall. In order to
45 optimize fish welfare and performance, aquaculture breeding companies are
46 performing intensive selection with constantly improving genetics and genomics
47 methods. The products of these costly programs represent the main asset of the
48 breeding companies, which is poorly protected from IPR violations.
49 A solution to all above-mentioned drawbacks would be the cultivation of reproductively
50 sterile fish. A traditional method for large scale sterilization is inducing triploidy in
51 fertilized eggs(Benfey, 2001). However, after careful reassessment of the pros and
52 cons, the method is no longer recommended neither by the Norwegian Fish Farmers
53 association (FHL) nor by the Norwegian Directorate of Fisheries (letter to Directorate
54 for Nature Management, 2021).
55 Ablation of the primordial germ cells (PGC) appears as an appealing alternative for
56 achieving fish sterility. Recently, a targeted CRISPR/Cas mediated knock out (KO) of
57 *dead end (dnd*) encoding a crucial germ cell-specific RNA-binding molecule resulted
58 in sterile Atlantic salmon(Wargelius et al., 2016). Using external *dnd* mRNA to rescue
59 the migrating early PGCs, the authors are trying to develop this CRISPR-based
60 approach to impose inherited sterility(Güralp et al., 2020). Nevertheless, the strategy
61 is based on DNA manipulation and, according to the existing regulatory work frames,
62 results in GMO fish that is currently unsuitable for farming and human consumption.
63 As an alternative to the inherited gene manipulation, transient gene downregulation
64 can be achieved with antisense oligonucleotides (ASOs). Genetic downregulation of
65 genes important for the PGCs development have been used to induce sterility in
66 various fish species, using species-varying methods from transient gene knock down
67 (KD) with morpholino oligonucleotides (MO) to mutants bearing gene KO mutations in
68 the relevant genes (reviewed in(Wong and Zohar, 2015a)). MOs complementary bind
69 the targeted mRNA, thereby preventing its translation or splicing(Heasman, 2002). In
70 addition to such passive inhibition, miRNA and siRNA can trigger mRNA degradation

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71 by activation of the RISC complex(Valencia-Sanchez et al., 2006). mRNA degradation
72 can also be initiated by Ribonuclease H (RNase-H), when single-stranded DNA oligos
73 bind to the mRNA and form DNA-RNA complex. The latter ASOs can be mimicked with
74 Gapmers (GAPs), synthetic ASOs consisting of RNA and bridged DNA (LNA)
75 ribonucleotides(Crooke et al., 2021). RNase-H degradation is enzymatic with the
76 recycled GAP oligos priming the reaction. Hence, each single GAP molecule can
77 trigger the degradation of multiple copies of the target RNA, whereas a single steric-
78 blocking MO can only inactivate one target RNA molecule.
79 In this work, we used MOs and GAPs for downregulation of crucial PGC genes in
80 Atlantic salmon. We find that MOs only transiently reduced the PGC numbers and
81 failed to ablate this cell lineage during 300-500 degree days (DD) of embryonic
82 development. In contrast, Gapmers induced degradation of the targeted mRNA and
83 successful gene KD. Salmon embryos injected with GAPs targeting *dnd* showed
84 absence or strong reduction of PGCs numbers and developed into sterile fish with
85 strongly reduced gonads without gametes. The performance of the sterile fish was
86 followed until adulthood and published in (Tveiten et al., 2022).
87
88 **Results**
89
90 **Injections of MOs targeting PGC-specific genes did not lead to PGC ablation**
91 Over the course of multiple experiments, we have designed and microinjected MOs
92 targeting *dnd, dazl, tudor 7 (tdr7b)* and *ziwi* (piwi-like1) genes in fertilized salmon eggs.
93 To determine the maximal dose of morpholino, we injected 100 eggs with each
94 individual oligo in concentrations 0.5mM, 0.4mM, and 0.25mM and evaluated the
95 mortality and the developmental defects induced by the reagent. These concentrations
96 were then refined in 0.1mM steps to determine the highest concentration at which the
97 mortality was in the same range as the non-injected controls and only occasional
98 malformed embryos were observed. We fixed a fraction of the treated embryos, stained
99 the PGCs using *vasa* antisense probe (Fig. 1E, F) and quantified their numbers at 300
100 DD and 500 DD stages (Fig. 1B). Whereas some samples showed a reduction in the
101 numbers of PGCs at the 300 DD stage, we did not find any embryos completely
102 depleted from PGCs at 500 DD (Fig. 1B). In particular, we targeted salmon *dnd* gene
103 with two morpholinos (*dnd*MO1, *dnd*MO2) binding close to the ATG translation start
104 site, and a splice MO (*dnd*MO3) binding at the splice donor site of Exon1 and its

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105 adjusted intron (Fig. 1A and Table 1), with no significant effect on the PGC numbers at
106 500 DD.
107

108 **Germ cells ablation using Gapmer oligonucleotides**

109 As translational and splicing blocking morpholinos could not trigger PGCs ablation, we
110 set out to test ASOs employing RNase-H mediated mRNA degradation, targeting five
111 different regions of the *dnd* coding sequence (Fig. 1A). Previous research in zebrafish
112 have demonstrated that Gapmers (GAPs) are efficient in tenfold lower concentrations
113 than the morpholino oligos(Pauli et al., 2015). Based on our survival titration
114 experiment for the morpholino ASOs, we tested GAPs in 0.050mM, 0.020mM and
115 0.005mM concentrations. As reported in the zebrafish studies, higher concentrations
116 of the ASOs increased mortality rates of the injected embryos (Table 2). At the chosen
117 concentration ranges, *dnd*GAP5 appeared more toxic than the other four oligos,
118 implying sequence-related toxicity. For our further experiments, we chose GAPs
119 concentrations at which the survival rates of the injected embryos were comparable
120 with the ones of non-injected control, mortality most likely due to the unfertilized eggs
121 in the batch (Table 2). Examinations of the mRNA levels at 56 DD showed rapid
122 degradation of the targeted *dnd* mRNA already at this early developmental stage (Fig.
123 1D). With some variations, all five Gapmers caused reduction of the number of the
124 PGCs at both 300 and 500 DD, with up to 80% of the investigated embryos being
125 completely PGCs free in some groups (Fig. 1C, F).
126
127

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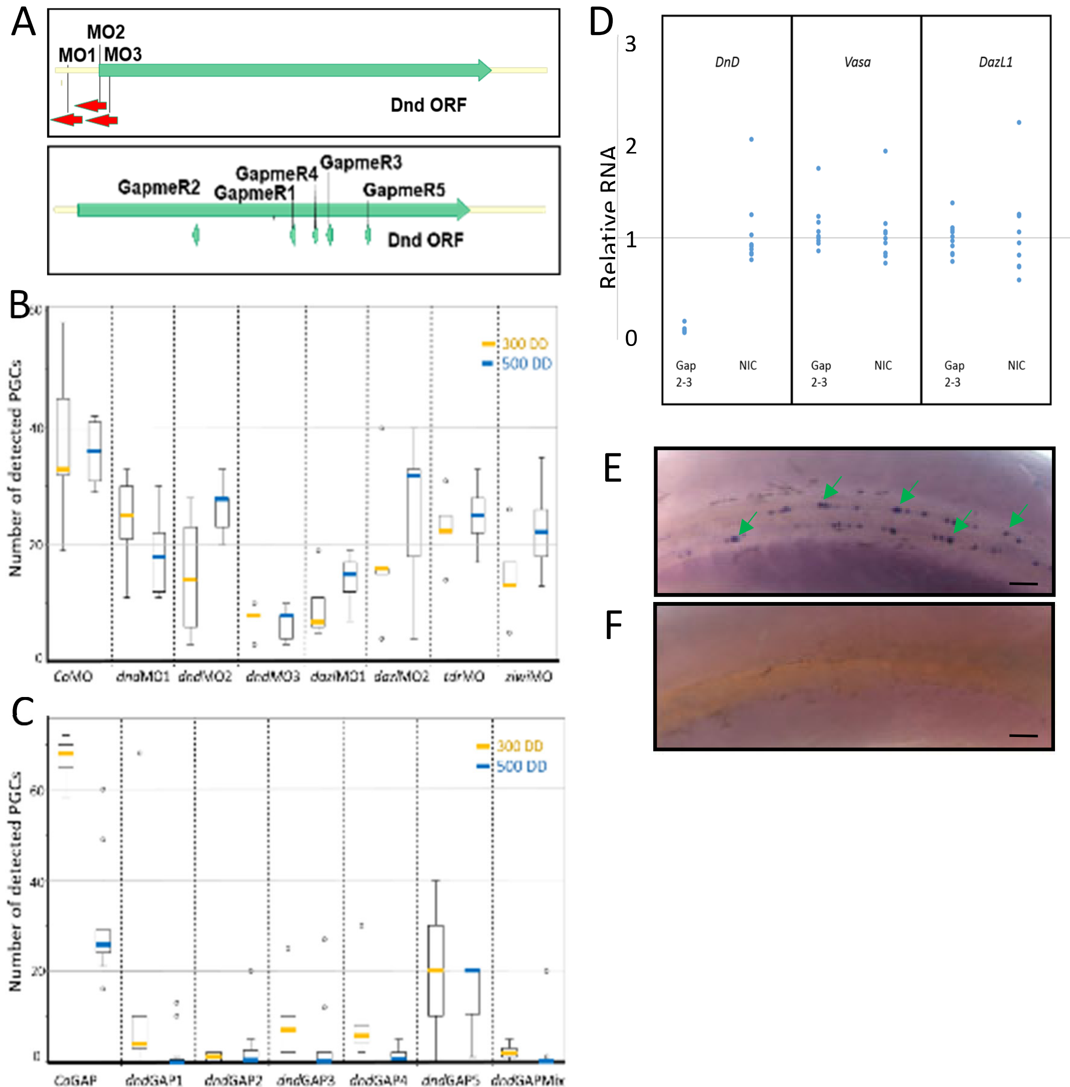

Figure 1. Gapmer, but not morpholino, ASOs targeting *dnd,*lead to gene knock down and germ cell ablation in salmon embryos. A) Schematic drawing of the regions of *dnd* mRNA targeted by the corresponding ASOs. B) Quantification of the PGC numbers in ten salmon embryos injected with MO ASOs targeting *dnd, dazl, tdr* and *ziwi*. No embryos completely depleted from PGCs were observed neither at 300 DD nor at 500 DD. C) Injections of five different GAP ASOs targeting the *dnd* gene individually and as a mix led to rapid decrease in the PGC numbers, rendering up to 80% (*dnd*GAP2) of the examined embryos (n=10/group) germ cell free at 300 DD and 500 DD stages. The values are presented as a box diagram, with whiskers found within the 1.5 IQR value and outliers outside this region depicted as individual points. D) Specific degradation of *dnd* mRNA mediated by *dnd*GAP2 at 56 DD stage. The levels of the PGC-specific *vasa* and *dazl* mRNAs remained unaltered by the treatment. (NIC

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141 – non-injected control). E, F) Example photographs of *vasa* WISH stained 300 DD
142 embryos, control (E) and *dnd*Gap2 injected (F). The germ cells are clearly visible in
143 the control (green arrows) and absent in the *dnd* KD samples. Scale bars, 100µm.
144

145 **PGC depleted salmon embryos grew into morphologically normal sterile fish**

146 To investigate the effect of Dnd depletion on the fish development, we injected more
147 than 2000 eggs with *dnd*Gap2 and raised the resulting embryos to juvenile stages
148 (around 110g) and to adults. At these stages, randomly sampled fish injected with
149 *dnd*GAP2 were externally morphologically undistinguishable from their control siblings
150 (Fig. 2A). A detailed comparison of the production performance of the sterile and fertile
151 groups was recently published in (Tveiten et al., 2022).
152 Dissections of randomly sampled control fish (Fig. 2 B, D) revealed, roughly even
153 distribution between the two sexes (n=153, and (Tveiten et al., 2022)). Sterile and
154 fertile female gonads displayed striking difference between a prominent orange
155 structure and a faint translucent string, respectively, (Fig. 2B), while the morphological
156 differences between the testes in sterile and fertile fish were less obvious (Fig. 2C).
157 WISH staining of the gonads with *vasa* AS probe demonstrated complete depletion of
158 the PGC population in the *dnd* KD fish, in contrast to the intact fertile fish (Fig. 2D-G).
159 Microscopy as well as histological examinations allowed us to determine the sex of the
160 empty gonads, confirming that the PGCs depletion did not alter the equal female to
161 male distribution. The percentage of sterile fish sampled at different stages varied
162 between 88% and 98%. This corresponds well with the proportion of WISH stained
163 embryos completely devoid of PGCs at 300-500 DD, and suggest that the germ cell
164 line may not have the capacity to re-generate beyond this stage.
165

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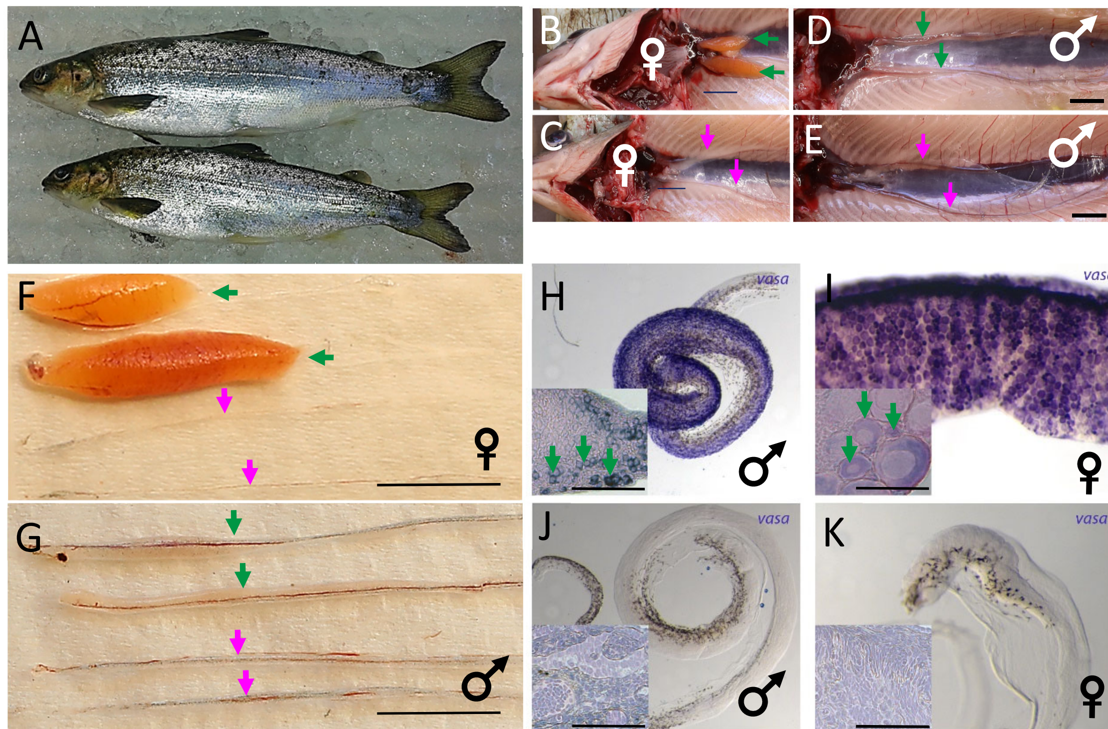
166 

167

168 **Figure 2. Germ cells depleted embryos develop into morphologically normal,**
169 **sterile fish.** A) A representative photo of eight-months old *dnd*GAP2-treated (up) and
170 control (down) juvenile salmon. Dissections of a female (B, C) and male (D, E) wild
171 type and sterile juvenile fish. Green arrows indicate the normal and magenta, the
172 rudimentary gonads. Scale bars 10mm. F, G) Magnified photos of the dissected
173 gonads from the previous panels. Scale bar 10mm. H-K) WISH with *vasa* AS probe of
174 male and female gonads from control (H, I) and *dnd* Gapmer-treated (J, K) juvenile
175 fish. Insets in each panel show histological cross sections of the samples at higher
176 magnification. The green arrows point to *vasa*-stained primary spermatogonia in (H)
177 and developing oocytes in (I). No PGC-specific staining was detected in the sterile
178 ovaries (J) and testes (K). Scale bar 100 um.

179

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180 **Discussion**

181 Antisense oligonucleotide technology is a powerful tool for altering gene expression in
182 research and medicine (Oberemok et al., 2018; Roberts et al., 2020). In this work, we
183 demonstrate ASO-mediated degradation of the germ cell-specific *dead end* transcript
184 in Atlantic salmon, leading to the ablation of the PGCs lineage and resulting in sterile
185 animals. We achieved successful gene KD by using Gapmer ASOs employing the
186 RNase-H degradation pathway, but not with ASOs of the morpholino type. Due to their
187 high efficiency, stability and relatively low cost, morpholinos have been the preferred
188 ASOs used in fish research. In zebrafish, MO-mediated KD of *dnd, nanos3,* and *ziwi*
189 led to PGC ablation and complete or partial infertility(Houwing et al., 2007; Weidinger
190 et al., 2003), and *dnd* KD in goldfish, starlet, common carp and loach had similar
191 effects(Fujimoto et al., 2010; Goto et al., 2012; Linhartová et al., 2015; Tao et al., 2022).
192 Although there have been some reports on successful use of MOs in
193 salmonids(Yoshizaki et al., 2016), in our hands, none of the tested oligos could trigger
194 gene knockdown and germ cell ablation in Atlantic salmon embryos. As MO actions
195 are known to be sequence-dependent, the simplest explanation of unsuccessful or
196 compromised MO effects could be that none of the oligos used here were specific
197 enough to trigger the complete KD effect. Nevertheless, we targeted *dnd,* a gene
198 proven to be indispensable for the PGCs survival (Wargelius et al., 2016), with three
199 different MOs, including the ATG region. Although this does not exhaust the potential
200 targeting sites in the mRNA, manufacture-designed MOs for similar studies in zebrafish
201 are usually very efficient. Alternative explanations for the lack of MO effect might be
202 that salmonids develop at low temperature of 4-8 °C with relatively long ontogeny.
203 Albeit at lower temperature, the MOs need to remain biologically active over a long
204 period, which might be leading to compromised gene KD. In addition, the injected
205 reagent volume, relative to the total egg volume, is far lower in salmon eggs than in
206 zebrafish and increasing reagents concentration leads to developmental defects. In
207 summary, transient reduction of germ cells numbers in some of the MO injected
208 embryos, suggests that the biological activity of the injected MOs was insufficient for
209 sustained gene KD over the entire embryonic development. The PGC recovery
210 observed at later stages can be attributed to proliferation of the germ cells not affected
211 by the treatment. Gapmers triggering mRNA degradation executed by the endogenous
212 enzymes, adapted over the course of evolution, appear to be a more potent silencing
213 tool, as demonstrated in other studies(Pendergraff et al., 2017). *dnd* mRNA, in

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214 particular, is maternally deposited in the oocyte, and the rapid degradation of these
215 mRNAs before the onset of endogenous transcription is certainly advantageous for the
216 successful gene KD.
217 Sterile salmon produced using this molecular technology were morphologically
218 undistinguishable from their fertile siblings. Previous experiments with *dnd* KO using
219 CRISPR(Wargelius et al., 2016) revealed that, unlike zebrafish(Slanchev et al., 2005),
220 salmon embryos depleted from the PGCs retained sexual identity and developed either
221 male or female gonads. Our work confirms that the sex determination in salmon is
222 uncoupled from the germ cell presence and PGC ablation method. Interestingly,
223 CRISPR generated sterile fish failed to undergo puberty and did not produce sexual
224 hormones upon stimulation(Kleppe et al., 2017). GAP sterilization did not hinder
225 gonadal sex steroid synthesis in the early maturing males(Tveiten et al., 2022).
226 However, the circulating sex steroid concentrations decreased to basal levels after
227 seawater transfer that might indicate termination of sexual maturation in the GAP
228 sterilized fish as well.
229 An efficient large-scale method for delivering oligonucleotides would be crucial for
230 potential aquaculture application of ASO-mediated fish sterilization. In this work, ASOs
231 were delivered into fertilized salmon eggs through manual microinjections, a laborious
232 method prone to errors. Correspondingly, we observed fluctuations in the success rate
233 of the treatment, which we attribute to imperfect delivery. GAP ASOs appear to be very
234 potent in specifically degrading the targeted mRNA (Fig. 1 and (Pauli et al., 2015)).
235 Their catalytic-like mechanism of action permits effective usage at very low cellular
236 concentrations. In addition, due to the chemical modifications, GAPs are resistant to
237 nucleases and highly resilient to degradation(Crooke et al., 2021). These features
238 could be utilized in the development of protocols for ASOs delivery with incubation of
239 eggs and sperm in solutions with active ASOs. An interesting study in 2015 reported
240 on successful delivery of modified MOs to zebrafish eggs with a bath incubation(Wong
241 and Zohar, 2015b). Further, methods developed for targeted drug delivery with various
242 nanocarriers as vehicles might also be successful as delivery strategies targeting
243 gonads, unfertilized eggs and sperm (Reviewed in(Edis et al., 2021)). The high
244 potency, specificity, relatively low cost, and non-GMO nature of action place Gapmers
245 among the top candidates for sterilizing agents provided a successful large-scale
246 delivery protocol is established.

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249 # Methods

250 ## Experimental procedures

251 Salmon eggs were produced under commercial settings by AquaGen, Norway and
252 provided for the experiments. One-cell stage fertilized eggs were aligned in a custom-
253 made setup and microinjected into the cell using pressurized microinjector from World
254 Precision Instruments. Injection volumes were optically adjusted to about 5% from the
255 cell volume. Injected eggs were incubated at 4-8°C to 56, 300 and 500DD stages and
256 sampled for the experiments. More than 2000 control and *dnd*GAP2 injected embryos
257 were transferred after hatching to standard hatchery conditions (continues light, food
258 in excess and temperature varying from 4-12°C and reared there until 8 months of age,
259 when a fraction was sampled for gonads inspection and histology. Throughout the
260 experiment fish were sampled and anaesthetized in Benzoak™-Vet (ACD Pharma),
261 containing 20% w/w Benzocaine as active substance, and killed using an overdose
262 (1 ml/L) (see also (Tveiten et al., 2022).

263 The sex of the juvenile fish was determined under a stereo microscope, after abdominal
264 section and removal of the internal organs. The differences between fertile and sterile
265 fish from both sexes is shown in Fig. 2.

266

267 ## RNA extraction and complementary DNA synthesis

268 Total RNA was extracted using conventional TRIzol method as described in (Kleppe et
269 al., 2015). Briefly, eggs were homogenized in a TissueLyser (Qiagen) using steel
270 beads with Trizol (Invitrogen). Homogenized samples were treated with chloroform,
271 and RNA was precipitated with isopropanol, washed with 80% ethanol and dissolved
272 in nuclease-free water. Genomic DNA contaminant was removed with DNase
273 treatment using TURBO DNAfree TM Kit (Thermo Fisher Scientific) according to the
274 manufacturer protocol. The quality and concentration of the RNA were determined
275 spectrophotometrically by Nano Drop (Nano Drop Technologies). The measured
276 A260/A280 ratio of 1.9–2.0 indicated high purity RNA.

277 RNA samples were reverse-transcribed with High-Capacity RNA-to-cDNA™ Kit
278 (Thermo Fisher Scientific) using 200 ng RNA in 20 µl reaction volumes The reaction
279 was incubated in a thermocycler for 37°C for 60 min and stopped by heating at 95°C
280 for 5 min before hold at 4°C. The synthesized complementary DNA (cDNA) was diluted
281 1:40 and used as a template for qPCR analysis.

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282

283 **qPCR analysis**

284 qPCR was used to measure relative expression of PGC-related genes. Specific
285 primers were designed using Primer blast (NCBI) and Integrated DNA Technologies
286 (Table 1). The amplification efficiency of each primer pair was calculated using a
287 twofold dilution series of a cDNA mixture according to the equation: E = 10 (−1/slope)
288 (Pfaffl, 2001). The melting peak for each amplicon was inspected to check for
289 unwanted amplification products. A control reaction to verify the absence of genomic
290 DNA was conducted on three randomly selected RNA samples. The qPCR was run in
291 duplicates in 7500HT sequence Dection system (Applied Biosystems) using the
292 following recommended parameters: Standard run mode with 40 cycles at 50°C for
293 2 min, 95°C for 10 min, and 60°C for 1 min. Following by the melt curve stage at 95°C
294 for 15 s, 60°C for 1 min, and 95°C for 15 s. Ct threshold was set between 0.1 and 0.2.
295 Each well contained Fast SYBR Green PCR Master Mix, 500 nM final concentration of
296 each primer, 5 µl diluted cDNA (1:40) and nuclease free water (Ambion) to a final
297 reaction volume of 15 µl. All data were collected by the 7500 Software and Analysis
298 Software (Applied Biosystem) and exported to Microsoft Excel for further analyses. The
299 Pfaffl method was used to calculate relative expression (Pfaffl, 2001). The geometric
300 mean (Anstaett et al., 2010) of the three reference genes β1-actin, elongation factor
301 1α (ef1α), and ribosomal protein 18S were used to normalize the gene expression and
302 remove nonbiological variation. Values from the control fish was used as calibrator as
303 denoted by Pfaffl(Pfaffl, 2001).

304

305 **Reagents**

306 All ASOs sequences can be found in Table 1. *dazl* (XM_014178361), *tudor7b*
307 (XM_014207215), *ziwi (piwi-like*, XM_014171836)
308 All morpholino ASOs were designed by GeneTools prediction software based on the
309 Salmon Genome Assembly at NCBI.
310 Gapmer ASOs were designed by Exicon/Quiagen prediction software, based on the
311 CDS of Atlantic salmon *dnd1* gene (acc. JN712911). The mimics were composed of
312 2'-OMe locked nucleic acids (LNA) and phosphorothioated (PS) of the DNA
313 backbone (Exicon/Quiagen).

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314 Lyophilized ASOs were dissolved in nuclease free water to 100µM stock solution.
315 The final concentration in the injection mix is indicated in Table 1, diluted in 1x
316 Danieau's solution with 0.5% Phenol Red.
317

318 **Whole mount *in situ* hybridization (WISH) and histology**

319 WISH of salmon embryos was performed as described in(Nagasawa et al., 2013) using
320 1kb fragment from the cDNA of the salmon *vasa* gene (JN712912) as a probe. For
321 histological analyses, gonads from juvenile fish were dissected out, fixed in 4% PFA
322 overnight and subjected to the same WISH procedure as the embryos. Subsequently,
323 the tissue was dehydrated, embedded in Technovit 7100 (Electron Microscopy
324 Sciences), sectioned at 6µm thickness, and conter-stained with Eosin according to
325 conventional histological procedures. The sections were then imaged at a Zeiss
326 Axioplan microscope at 20x magnification.
327
328

329 **Ethical statements**

330 The experimental protocols were planned according to the ARRIVE guidelines and
331 approved by the Norwegian Food Safety Authority (FOTS permit ID 17389), in
332 accordance with the animal welfare law of Norway and the instruction for use of
333 animals for research https://www.forskningsetikk.no/en/guidelines/science-and-
334 technology/ethical-guidelines-for-the-use-of-animals-in-research/
335

336 **Data availability**

337 The data generated and/or analyzed in the current study are provided in the paper.
338

339 **Funding**

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341 BIOTEK2021 project SALMOSTERILE (221648).

342

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343

344 **Table 1. ASOs sequences (5' to 3' direction)**

ASO	Sequence
T blocking dnd MO1	ACAATTACCGAAACTGATTTCTAGT
T blocking dnd MO2	CTGACTTGAACGCTCCTCCATTATC
S blocking (Ex1-Int1) dnd MO3	GTTATGCTATTTCAGTTTACCTGAC
T blocking *dazl* MO	GAATAAATTAAACACACTGCCGCGC
T blocking *tdr7b* MO	TTCTTCATCAGATCGTCAGACATCC
T blocking *ziwi* MO	GCCAGTCATTTTCAGCTTCTAAAAC
GAP sequences	
dnd GAP1	GCCAGCCCGAAGCGTT
dnd GAP2	AACGAGCGGATGGCGG
dnd GAP3	GAGCAGCGAGGAGGTT
dnd GAP4	TTGGAGGGTCTGGGAG
dnd GAP5	GATTCAGGAGCAGCGT
RT PCR Primers	
*dnd*F	CACAAGGAGGGAGCAACTG
*dnd*R	GCACAAGGAGGGAGCAACTG
*vas*F	CCAGTACAGAAGCATGGCATTC
*vas*R	CCGTTTTCCCAGATCCAGTCT
*dazl*F	TGTTGGAGGAATCGACATG
*dazl*R	TGAGTACAGGTGAGGGCTG

345

346 Table 2. Survival rates of Gapmer injected embryos.

Gapmer	Conc. (μM)	Dead	Alive	Total	Survival (%)
dndGAP1-1	50	79	10	89	11.2
dndGAP1-2	20	23	67	90	74.4
dndGAP1-3	5	30	65	95	68.4
dndGAP2-1	50	78	5	83	6
dndGAP2-2	20	63	15	78	19.2
dndGAP2-3	5	17	66	83	79.5
dndGAP3-1	50	84	0	84	0
dndGAP3-2	20	66	8	74	10.8
dndGAP3-3	5	28	61	89	68.5
dndGAP4-1	50	19	64	83	77.1
dndGAP4-2	20	19	66	85	77.6

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dndGAP4-3	5	20	58	78	74.4
dndGAP5-1	50	72	0	72	0
dndGAP5-2	20	68	8	76	10.5
dndGAP5-3	5	55	20	75	26.7
Control	0	16	69	85	81.2

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