Impact of germ cell ablation on the activation of the brain–pituitary–gonadal axis in precocious Atlantic salmon (*Salmo salar* L.) males

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Abstract
The germ cells are essential for sexual reproduction by giving rise to the gametes, but the importance of germ cells for gonadal somatic functions varies among vertebrates. The RNA-binding dead end (Dnd) protein is necessary for the specification and migration of primordial germ cells to the future reproductive organs. Here, we ablated the gametes in Atlantic salmon males and females by microinjecting *dnd* antisense gapmer oligonucleotides at the zygotic stage. Precocious maturation was induced in above 50% of both germ cell-depleted and intact fertile males, but not in females, by exposure to an off-season photoperiod regime. Sterile and fertile males showed similar body growth, but maturing fish tended to be heavier than their immature counterparts. Pituitary *fshβ* messenger RNA levels strongly increased in maturing sterile and fertile males concomitant with the upregulated expression of Sertoli and Leydig cell markers. Plasma concentrations of 11-ketotestosterone and testosterone in maturing sterile males were significantly higher than the basal levels in immature fish, but lower than those in maturing fertile males. The study demonstrates that germ cells are not a prerequisite for the activation of the brain–pituitary–gonad axis and sex steroidogenesis in Atlantic salmon males, but may be important for the maintenance of gonadal somatic functions.

KEYWORDS
androgens, dead end, germ cells, Leydig cells, puberty, Sertoli cells

1 | INTRODUCTION

The development of primordial germ cells (PGCs) into gametes is an essential feature of sexual reproduction in animals (Hansen & Pelegri, 2021; Johnson & Alberio, 2015). During early embryogenesis, the PGCs migrate to the genital ridges and undergo meiosis to generate oocytes or sperm in the developing ovary or testis (Richardson & Lehmann, 2010). Whereas signals from gonadal somatic cells are universally needed to direct germ cell development, the requirement for germ cells in gonadal development and maintenance of tissue integrity...
seems to vary among vertebrates (DeFalco & Capel, 2009; Rios-Rojas et al., 2015). The current view is that germ cell signaling is important for fetal ovary development, but is not required for testis development, although many conflicting studies exist (Rios-Rojas et al., 2015). For example, germ cell-depleted female mice showed no defects in the ovarian somatic function and the steroidogenic function was maintained despite significantly reduced gonadotropin follicle-stimulating hormone (Fsh) levels (Maatouk et al., 2012; McNelly et al., 2000). Male mice lacking germ cells had decreased number of Sertoli cells and upregulated testicular expression of androgenic enzymes suggesting abnormal high testosterone production (Rios-Rojas et al., 2016). In the red-eared slider turtle (Trachemys scripta), removal of germ cells by busulfan did not influence sex determination or morphological differentiation of the fetal gonads (DiNapoli & Capel, 2007), while the germ cells in Xenopus laevis are crucial during ovarian development, but are not necessary for testis formation (Piprek et al., 2012). In teleost fish, the importance of germ cells for the gonadal sex differentiation varies between species. Germ cells are a prerequisite for female development in zebrafish (Danio rerio), medaka (Oryzias latipes), and mackerel (Scomber australasicus) as ablation of PGCs results in phenotypic, though sterile, males only (Kawamura et al., 2020; Kurokawa et al., 2007; Siegfried & Nüsslein Volhard, 2008; Slanchev et al., 2005; Weidinger et al., 2003). In comparison, PGC ablation failed to affect the sexual fate of gonadal somatic cells in loach (Misgurnus anguillicaudatus), goldfish (Carassius auratus), grass puffer (Takifugu albopombeus), and salmons, which could develop as either phenotypic males or females (Fujihara et al., 2022; Fujimoto et al., 2010; Goto et al., 2012; Wargelius et al., 2016; Yoshikawa et al., 2020; Yoshizaki et al., 2016). In zebrafish, removal of germ cells led to incomplete masculinization of the brain despite normal sex steroid levels (Pradhan & Olsson, 2018), while dnd-knockout Atlantic salmon (Salmo salar) males and females showed basal plasma sex steroid concentrations and low gene expression levels along the brain–pituitary–gonadal (BPG) axis at the seawater stage (Kleppe et al., 2017). Hence, the germ cells were concluded to be required for pubertal activation of the gonadal steroidogenesis in Atlantic salmon.

Similar to other vertebrates, the reproduction cycle of teleosts is regulated by the BPG axis, whereby the gonadotropin-releasing hormone (GnRH) stimulates the production of the pituitary gonadotropins Fsh and luteinizing hormone (Lh), which regulate the gametogenesis and steroidogenesis (Weltzien et al., 2004; Wootton & Smith, 2014). Most vertebrates synthesize two or three GnRH variants of which GnRH3, or salmon GnRH, seems to be the main activator of gonadotropin secretion in salmonids (Amano, Ikuta, et al., 1997; Dickey & Swanson, 2000). In teleosts, Fsh is the main gonadotropin in early stages of maturation and regulates spermatogenesis and sex steroidogenesis in males by controlling Sertoli and Leydig functions (Campbell et al., 2003; Gomez et al., 1999; Planas & Swanson, 1995; Prat et al., 1996; Sohn et al., 1999). Fsh activates the release of the teleost-specific insulin-like growth factor 3 (IGF3) from Sertoli cells (Li et al., 2021; Nóbrega et al., 2015), and suppresses the production of anti-Müllerian hormone (Amh) exerting inhibitory effects on spermatogenesis and steroidogenesis (Morais et al., 2017; Pfennig et al., 2015; Skaar et al., 2011). Leydig cells are the primary source of androgens, and the synthesis requires the coordinated action of conserved steroidogenic enzymes (Miller, 1988). 11-Ketotestosterone (11-KT) is the major sex steroid in all stages of spermatogenesis in teleosts, while testosterone (T) works through positive feedback mechanisms on the hypothalamus and pituitary (Amano, Ikuta, et al., 1997; Borg, 1994; Rege et al., 2019).

Anadromous salmonids exhibit high plasticity in life history strategies and the males show extreme variability in age and size at puberty (reviewed by Mobley et al., 2021). Salmonid males can mature already at the parr stage, during smoltification in freshwater or as postsmolt after seawater transfer, while maturation in females commonly occur at the postsmolt stage in the sea (Baum et al., 2005; Fjelldal et al., 2018; Fleming, 1996; Heinimaa & Erkinaro, 2004; Klemetsen et al., 2003). The percentage of precocious males varies widely in different populations, ranging from completely absent to all individuals sampled and is likely caused by environmental and genetic factors (Mobley et al., 2021). Since not all males usually undergo early maturation, Atlantic salmon is an excellent model for studying pubertal changes in the BPG axis in fish of same age and origin. Early maturation is a common problem in salmon aquaculture by compromising somatic growth, harvest quality, and fish welfare, and farmed escapees might threaten wild salmon populations (Bolstad et al., 2021; Taranger et al., 2010). These problems could at least partly be solved by producing sterile salmon lacking germ cells at large scale using the bath immersing technology established in zebrafish (Wong & Zohar, 2015). However, possible effects on gonadal sex steroid levels, somatic growth, and behavior need to be evaluated before introducing germ cell-free fish in aquaculture. Here, we examined the requirement of germ cells for the activation of the BPG axis and functions of the gonadal somatic cells in Atlantic salmon by comparing germ cell-depleted and intact fertile males comprising both immature and early maturing individuals at the freshwater stage.

2 | RESULTS

2.1 | Absence of PGC markers and gametes in dnd-knockdown salmon

Microinjection of dnd-antisense gapmers in fertilized Atlantic salmon eggs resulted in undetectable messenger RNA (mRNA) levels (Ct > 33) of dnd and vasa in 92% of the testes and ovaries examined about 2 years after the treatment. All fish in this study was evaluated for gonadal expression of dnd and vasa, and only those with undetectable levels were considered as sterile fish. In comparison, both germ cell markers were expressed in the intact gonads of the fertile males and females with significantly higher dnd transcript levels in the females and with a similar, although not statistically significant, sex divergent trend for vasa (Figure 1). Neither dnd nor vasa had any clear temporal expression pattern.

Evaluation by histology and immunohistochemistry revealed that the string-like gonads of the dnd-knockdown males and females were lacking gametes and no Vasa protein were visible (Figure 2a,b).
FIGURE 1  Temporal changes in gonadal relative expression of the germ cell marker genes *dnd* (a) and *vasa* (b) in fertile female (♀ *n* = 8–16) and fertile male (♂ *n* = 9–16) Atlantic salmon during exposure to continuous light for 0 (WK 0), 3 (WK 3), and 6 (WK 6) weeks at 10°C in freshwater. Before long day treatment, all fish were exposed to a short-day photoperiod of 6 h of light (L) and 18 h of dark (D) (6L:18D) for 8 weeks. Box-whisker plots show medians, minimum, and maximum values (whiskers) and the interquartile range (box) of the values. Dots outside whiskers are data "outliers." Differences between groups within samplings are indicated by different lowercase letters (*p* < 0.05). Differences across samplings within groups are indicated by different uppercase letters (*p* < 0.05). For clarity, letters are omitted when no significant differences were found within datasets.

FIGURE 2  Immunohistochemical analysis of germ cell-specific Vasa (red staining) in Atlantic salmon gonads. (a) *dnd*-knockdown male, (b) *dnd*-knockdown female, (c) fertile male with undifferentiated spermatogonia, (d) fertile male with differentiated spermatogonia, and (e) fertile female with previtellogenic oocytes as the most advanced stage of development. Negative controls are inserted. Scale bar = 60 µm.
Temporal changes in pituitary fshβ relative gene expression in immature fertile (n = 6–13), immature sterile (n = 8–11), maturing fertile (n = 9), and maturing sterile (n = 9) male Atlantic salmon during exposure to continuous light for 0 (WK 0), 3 (WK 3), and 6 (WK 6) weeks at 10°C in freshwater. Differences between groups within samplings are indicated by different lowercase letters (p < 0.05). Differences across samplings within groups are indicated by different uppercase letters (p < 0.05). For clarity, letters are omitted when no significant differences were found within datasets.


The testicular tubuli and Sertoli cells in the germ cell-free testes were further examined below, while the presence of granulosa cells in the germ cell-free ovaries was suggested by the high cyp19a1a expression (data not shown). The fertile males and females showed prominent Vasa staining of the germ cells in the morphologically normal testes and ovaries (Figure 2c–e). In the ovary, previtellogenic oocytes was the most advanced oocyte stage found in fertile females, while the intact testes were either dominated by undifferentiated or differentiated spermatogonia. This suggested that the fertile males comprised immature and early maturing individuals that was confirmed in the analyses of the BPG axis below.

2.2 Activation of BPG axis in sterile and fertile males

Pubertal changes in the expression of genes along the BPG axis in sterile and fertile fish were quantified by quantitative polymerase chain reaction (qPCR). Pituitary fshβ were expressed at low levels in both fertile and sterile males at the initiation (WK 0) and at WK 3, but after 6 weeks (WK 6), fertile males displayed a clear bimodal frequency distribution in fshβ mRNA levels. Those individuals belonging to the upper mode (fshβ relative expression >10, n = 9, or 60%) were evaluated as having initiated maturation, while those in the lower mode (fshβ relative expression <0.4, n = 6) were characterized as being immature fertile males. Sterile males displayed a very similar bimodal frequency distribution in fshβ mRNA levels and, by using the same “cut off” parameters as for fertile fish, they were classified as maturing (n = 9, or 53%) or immature (n = 8) fish. The high and low fshβ transcript levels in the maturing and immature sterile males, respectively, were not significantly different from their fertile counterparts. Based on the above, all fish sampled at WK 0 and WK 3 were classified as immature. The pituitary lhb mRNA levels were low during the study and showed no systematic variations between or within the different groups (p > 0.05, data not shown). Neither fertile nor sterile females displayed any significant increase or clear modalities in fshβ mRNA levels (p > 0.05; data not shown). This, together with histology sections showing previtellogenic oocytes as the most advanced stage of development of the intact fertile ovaries, and with plasma T and E2 concentrations below 1 ng·ml⁻¹ (which clearly did not change over time, p > 0.05; data not shown), all females were classified as immature. Females were hence omitted from any further evaluation of other indices of BPG-axis activation in this study.

The gnrh3a and gnrh3b paralogs were expressed at low levels in the brain at the initiation of the long day treatment (WK 0) and at WK 3, but both paralogs showed large individual variations in the maturing males at WK 6 (Figure 4). The expression of gnrhr4a and gnrhr4b showed no significant changes in the brain during the study, while the pituitary gnrhr4b levels were upregulated in immature fertile males at WK 6 (data not shown). Fertile and sterile males had similar gonadal fshr mRNA levels before the onset of puberty, but the expression was downregulated in the fertile males at WK 6 (Supporting Information: Figure S1).

2.3 Sertoli cell activity

The upregulated fshβ expression in the maturing sterile and fertile males was accompanied by downregulated amh expression in the maturing fertile males, but not in the sterile males displaying similar levels as the immature fish (Figure 5a). These changes occurred concomitantly with strongly increased testicular igf3 mRNA levels in both maturing sterile and fertile fish, while low levels were recorded in immature males (Figure 5b). To account for the relative over-representation of somatic cells in the germ cell-free testis, we calculated the ratio of amh and igf3 mRNA levels, which was strongly negatively associated with the fshβ mRNA levels and separated the four male groups at the final sampling point (Figure 5c).

The testicular structure was maintained in the germ cell-depleted males, but the testicular tubuli were much thinner and contained less Sertoli cells compared to the intact testis (Figure 6, Table 1). The tubular cross-sectional area and the number of Sertoli cells increased significantly in the fertile, but not in the sterile, males during maturation.

2.4 Leydig cell activity

Plasma 11-KT and T concentrations increased in both sterile and fertile males during early maturation, but the fertile individuals showed significantly higher levels than the sterile fish at the final sampling point (Figure 7a,b). The immature males had low androgen levels of about 1 ng·ml⁻¹ throughout the study. We further examined
the steroidogenic capacity of fertile and sterile males by evaluating the gene expression of key androgenic enzymes (Supporting Information: Figure S2a). Both sterile and fertile males showed elevated mRNA levels of the key steroidogenic acute regulatory protein (StAR) throughout the study without any changes at the onset of maturation ($p > 0.05$). CYP17a1 (P450c17) catalyzes the conversion of the progesterone to androstenedione, and the coding cyp17a1a gene was expressed at significantly higher levels in the sterile than in the fertile males during maturation (Supporting Information: Figure S2b). CYP11B1 (11β-hydroxylase) and 11β-HSD catalyze the conversion of T to 11-KT. Fertile males expressed cyp11b1 at significantly higher levels than sterile males at WK 0 and...
WK 3, but the levels tended to decrease in both groups over time and did not differ between any of the groups at final sampling (p > 0.05, Supporting Information: Figure S2c). Expression of 11β-hsdB2 did not change over time and was not influenced by initiated maturation (p > 0.05, Supporting Information: Figure S2d). The Leydig cell marker wnt5a was expressed at high levels in both immature and maturing sterile males at final sampling point compared to the fertile counterparts, whereas the insl3b transcript levels were significantly increased in the fertile males, particularly during maturation (Figure 8a,b).

### 2.5 | Body biometrics

The germ cell-depleted sterile males had lower gonadosomatic index (GSI) than the fertile males, except at the first sampling point, and mean GSI increased significantly during maturation in fertile, but also in sterile males and was 0.13% and 0.03%, respectively, at final sampling (Figure 9a). At this point, GSI differed significantly between the four male groups and was higher in immature fertile males than in maturing sterile individuals. Body weight increased from about 200 g to 350 g during the experiment (Figure 9b). Fertile and sterile males showed no significant difference in body weight, but the maturing fertile and sterile individuals tended to be heavier than their immature counterparts at final sampling point. Furthermore, the condition factor (CF) was similar in fertile and sterile males during the study, but maturing fertile and sterile males showed significantly higher CF than their immature counterparts at final sampling (Figure 9c).

### 3 | DISCUSSION

Transient inactivation of Dnd in Atlantic salmon embryos resulted in lifelong loss of the germ cells in the sterile males and females. Furthermore, the study revealed that the germ cells were not required for the activation of the BPG axis in precocious males exposed to an off-season photoperiod regime. Both sterile and fertile males showed increased gene expression of pituitary Fshβ and key Sertoli and Leydig cell-specific factors together with elevated plasma androgen concentrations, which are all hallmark indicators of puberty onset in salmonid males (Campbell et al., 2003; Maugars & Schmitz, 2008; Middleton et al., 2019; Schulz et al., 2019). In contrast to the disorganized ovarian structure, the germ cell-free testis formed a normal architecture with intact tubuli structure and functional somatic cells. However, the maturing sterile and fertile males showed important differences that may shed light on the interactions between germ cells and somatic cells. The Sertoli cell population were lower and did not increase in numbers in maturing germ cell-depleted testes when compared to the maturing fertile fish, in agreement with the reduced Sertoli cell numbers and tubuli cross section in mutant mice devoid of germ cells (Rios-Rojas et al., 2016). The Sertoli cells are able to support a limited number of germs cells, and Sertoli cell proliferation is the primary factor responsible for the

| Male subgroups | Sertoli cell number | Tubuli cross-sectional area (mm²) |
|----------------|---------------------|----------------------------------|
| Immature sterile | 9.6 ± 0.6a          | 0.6 ± 0.05a                      |
| Maturing sterile | 10.2 ± 0.6a         | 1.0 ± 0.07a                      |
| Immature fertile | 16.4 ± 0.9b         | 2.0 ± 0.34b                      |
| Maturing fertile | 19.4 ± 0.4c         | 2.9 ± 0.24c                      |

Note: Mean ± SEM, n = 5–6 within each group of fish. Different lowercase letters within columns indicate statistical difference (p < 0.05) between groups of fish.

![Figure 7](image-url) Temporal changes in 11-ketotestosterone (a) and testosterone (b) plasma concentrations (ng·ml⁻¹) in immature fertile (n = 5–12), immature sterile (n = 7–13), maturing fertile (n = 9), and maturing sterile (n = 8) male Atlantic salmon during exposure to continues light for 0 (WK 0), 3 (WK 3), and 6 (WK 6) weeks at 10°C in freshwater. Differences between groups within samplings are indicated by different lowercase letters (p < 0.05). Differences across samplings within groups are indicated by different uppercase letters (p < 0.05). For clarity, letters are omitted when no significant differences were found within datasets.
increase in testis size and sperm production in teleosts, similar to mammals (Meroni et al., 2019; Schulz et al., 2015). In teleosts, the igf3-mediated proliferation of Sertoli cells and differentiating divisions of spermatogonia was suggested to be coordinated by germ-Sertoli cell communication (Safian, Bogerd et al., 2018). Accordingly, the igf3 expression was upregulated in both maturing sterile and fertile males, but only the latter showed increased number of Sertoli cells during early maturation. In comparison, germ cell-signaling towards Sertoli cells was proposed in rat wherein the production of the Sertoli-specific growth factor glial cell line-derived neurotrophic factor was modulated by the density of undifferentiated spermatogonia (Johnston et al., 2011).

Fsh was proposed to trigger a balanced activation of self-renewal and differentiation of undifferentiated spermatogonia by the coordinated actions of Wnt5a, Igf3, Insl3, and Amh (Safian et al., 2019; Safian, Ryane, et al., 2018). In contrast to sterile males, the maturing fertile males displayed strongly upregulated insl3 expression that was correlated with the wnt5a transcript levels (Supporting Information: Figure S3). The low insl3 expression in the germ cell-depleted testis could be explained by the function of Ins13 acting as germ cell survival factor (Crespo et al., 2021). Moreover, the stimulatory effect of Fsh on insl3 mRNA levels was shown to be inhibited by Amh (Skaar et al., 2011), which was strongly downregulated only in the maturing fertile males. In mouse, germ cell-depleted males showed upregulated gene expression of Ins13 (Rios-Rojas et al., 2016), which through neofunctionalization has adopted an important role in testicular descent into the mammalian scrotum (Huang et al., 2012; Nef & Parada, 1999; Park et al., 2008). The Leydig cell-derived Wnt5a plays a conserved function by promoting the proliferation and accumulation of undifferentiated spermatogonia (Cantú et al., 2016; Safian et al., 2019; Safian, Ryane, et al., 2018; Yeh et al., 2011). In chicken, overexpression of wnt5a activated the Wnt signaling pathway.
regulating the differentiation of embryonic stem cells into spermato- 
gonal stem cells in vitro (He et al., 2018). We, therefore, suggest that 
the upregulated wnt5a expression in both immature and maturing 
sterile males is induced by a local feedback loop due to the absence 
of undifferentiated spermatogonia that is supported by the fine- 
tuned coordination of the Wnt signaling pathway in germ cells and 
somatic cells (Cantú et al., 2016).

Steroidogenic activity of the Leydig cells was demonstrated in 
the sterile salmon males by the upregulated expression of key 
steroidogenic enzymes and elevated plasma androgen concentrations 
during early maturation. The lower androgen levels in sterile than in 
fertile males are probably related to the negative feedback loops 
between sex hormones and Amh reported in Atlantic salmon and 
zebrafish (Melo et al., 2015; Pfennig et al., 2015; Skaar et al., 2011). 
The simple fact that GSI was much lower in maturing sterile fish (c. 
four times) than maturing fertile, even after accounting for germ cell 
loss, which appear to constitute 20%–25% of all cells in male testes of 
this stage (Gührap et al., 2020, for references), may also have 
contributed to reduced sex steroid production. Although the germ 
cells are evidently not indispensable for the activation of the gonadal 
steroidogenesis in salmon males, we found reduced plasma androgen 
concentration (less than 1 ng·ml⁻¹) in the maturing sterile males after 
seawater transfer, in contrast to high 11-KT levels up to 55 ng/ml in a 
fertile male with running milt (data not shown). This is consistent with 
the basal sex steroid levels measured in dnd-knockout salmon males 
in seawater and likely explains the contradicting conclusion that germ 
cells are required for sex steroid production in Atlantic salmon 
(Kleppe et al., 2017). We suggest that the maturing sterile males 
failed to complete maturation after seawater transfer as previously 
reported in fertile salmon males (Fraser et al., 2019) due to 
inadequate positive feedback exerted by the androgens on the 
pituitary gonadotropins (Antonopoulou et al., 1999; Borg et al., 1998; 
Fontaine et al., 2020).

The strong increase in fshβ mRNA levels in the maturing males 
was not accompanied by any significant upregulation of the brain 
gnRh3 paralogs. Accordingly, precocious musu salmon (Oncorhynchus 
masou) showed no changes in the salmon (s) GnRH content in 
telencephalon, while the pituitary concentrations of sGnRH and 
GTHs increased during maturation (Amano et al., 1994). Among the 
six GnrH receptor paralogs expressed in the Atlantic salmon 
pituitary, the expression of gnrhr4a (gnrhr2bb) was moderately 
upregulated concomitant with increased fshβ levels in maturing 
salmon parr, but was exclusively localized in lhβ expression cells 
(Cliaia et al., 2020). The present study revealed no differences 
between immature and maturing males in the pituitary expression of 
gnrhr4a and gnrhr4b, but the latter paralog tended to be upregulated 
in immature sterile males. Teleost GnRH isoforms seem to play 
various functions in the brain, pituitary, and gonads (Amano et al., 1992; Soverchia et al., 2007) that should be further examined, 
including spatio-temporal expression studies of the different ligands 
and receptors. It should be noted that the increased fshβ expression 
ocurred in the sterile and fertile salmon males without any 
upregulation of the gonadal fshr levels. Accordingly, the receptors 
for gonadotropin and androgens did not seem to be relevant for the 
entry into puberty in precocious salmon males, but were already 
present and ready to respond to their ligands (Schulz et al., 2019).

Both sterile and fertile maturing males in this study tended to 
grow better and had higher CF than their immature counterparts. In 
salmonids, energy stores and the rate of energy acquisition during 
spring seem to be important determinants of whether an individual 
will mature in the following autumn (Dutil, 1986; Good & 
Davidson, 2016). In salmon aquaculture, faster growth in maturing 
than in immature fish during the early part of the reproductive cycle 
(before rapid gonad growth) (Åknes et al., 1986; Hunt et al., 1982; 
Rowe & Thorpe, 1990; Tveiten et al., 1996) is used to maximize 
biomass production. Thus, maintaining the ability of sterile males to 
mount a maturation induced growth response, despite later lack of 
gonad development, is commercially important and biologically 
interesting.

The endocrine mechanisms underlying the differences in body 
growth between maturing and nonmaturing salmonid fish are not 
well understood, but sex steroids (Berglund et al., 1992; Hunt 
et al., 1982; Tveiten et al., 1998; Youngson et al., 1988) and their 
possible influence on thyroid status (reviewed by Cyr & Eales, 1996) 
may be implicated. These studies corroborate well with findings in 
our study where elevated plasma sex steroid concentrations (c. 
2–10 ng·ml⁻¹) recorded in both sterile and fertile males appear 
positively correlated with body mass and CF.

4 | CONCLUSION

The Dnd gene silencing in Atlantic salmon resulted in the ablation of 
germ cells in males and females. The BPG axis was activated in 
precocious maturing sterile and fertile males displaying similar gene 
expression patterns that differed from the immature status of the 
nonmaturing males. Elevated plasma androgen concentrations and 
upregulated expression of key steroidogenic enzymes in both sterile 
and fertile males during early maturation strongly indicate that the 
germ cells are not a prerequisite for the activation of gonadal 
steroidogenesis. However, termination of maturation in the sterile 
males after seawater transfer was suggested due to reduced 
androgen levels. Furthermore, the number of Sertoli cells increased 
only in the maturing fertile males, indicating that germ-gonadal cell 
communication is essential for maintaining the gonadal functions.

5 | MATERIALS AND METHODS

5.1 | Fish and experimental setup

The study was conducted at the Aquaculture Research Station at 
Kårivika, Tromsø, in northern Norway using full sibling Atlantic salmon 
originating from egg and milt delivered by AquaGen breeding 
company. Germ cell ablation was achieved by microinjecting 
“Gapmer” antisense oligonucleotides targeting the dnd mRNA at
the one-cell stage in late October 2016. Microinjection was under-
taken according to Škugor, Slanchev et al. (2014) and Škugor, Tveiten et al. (2014) with slight modification. Briefly, one-cell stage
fertilized eggs were aligned in a custom-made setup and micro-
jected into the cell at a Gapmer concentration of 5 μM using a
pressurized microinjector (World Precision Instruments Ltd.). Injection
volumes were optically adjusted to about 5% of the cell volume.
Gapmers induce RNAse-H mediated mRNA degradation of the
targeted mRNA (reviewed by Crooke et al., 2021). The dnd-
 knockdown, referred to as germ cell-depleted or sterile, and intact
fertile fish were fed standard feed (Nutra ST, Nutra XP and Nutra
Olympic from Skretting AS) from the start-feeding stage and were
held under standard hatchery conditions (fed by disc feeders
24°C until August 2018. The fish were individually tagged with a
passive-integrated transponder tag in Week 34, and about 360 fish
totally were transferred into six 500-L freshwater tanks for triplicate
analysis. According to established protocols (Fjelldal et al., 2011;
Strand et al., 2018), smolitication was induced by exposing the fish
to a photoperiod of daily 6 h light (6L:18D) and water temperature
5–6°C for 8 weeks followed by continuous light and 10°C from
24.10. throughout the experiment. 10–12 fishes were randomly
sampled from each tank at three time points: after 0 (WK 0, 24.10),
after 3 (WK 3, 14.11), and after 6 weeks (WK 6, 05.12),
aesthetized in Benzoak™ and killed using an overdose (1 ml/L).
Blood was sampled from the caudal vasculature using vacutainer
tubes (Vacutainer™ containing 34 IU Li-heparin. Plasma was
pipetted off after centrifugation and frozen at −20°C until further
analyses. Body weight (nearest 0.5 g) and length (mm) were
recorded to calculate CF according to the formula (CF = weight
(g) × 100 × (length (cm)²)⁻¹. The fish was kept on ice before being
opened for dissection of brain, pituitary and gonads, which were
transferred to RNAlater (Ambion; 1:10 volume ratio) and stored at
−20°C after 4°C overnight. The gonads were weighed before being
bisedected with one part fixed in PFA for histology and immuno-
histochemistry, and the other part submerged in RNAlater for gene
expression analyses. GSI was calculated as gonad weight (nearest
0.001 g) × body weight⁻¹) × 100.

From August 2018, a subgroup of individually tagged sterile and
fertile fish was maintained under similar tank and feeding conditions
as described above, but exposed to natural photoperiod (69°39′N)
and temperature (3–12°C) in freshwater until smoltification in
April–May 2019. The fish were then transferred to full strength
seawater (34 ppt) and maintained at natural photoperiod and
temperature (3–10°C). In May 2020, they were moved to net pens
at the sea cage facility and held under the same photoperiod and
natural temperature conditions until sampling in October 2020.
Blood samples were taken from five fish of each group (see
Section 2) for measuring sex steroids as described above. At this
time, a 4-years-old Atlantic salmon held under culture conditions will
become sexually mature.

5.2 | Histology and immunohistochemistry

Formalin-fixed gonad tissues from sterile and fertile males (n = 6–10 per group) were processed overnight in Tissue
Processor (Logos; Milestone). Paraffin embedded tissues were
sectioned (2 μm) using a rotary microtome (Leica) and stained with
hematoxylin and eosin (Merck) in auto-stainer (Leica) at the
Norwegian Veterinary Institute, Harstad. All slides were then
analyzed at Nofima, Tromsø, using light microscopy and the QuPath
(Quantitative Pathology & Bioimage Analysis) software. Germ cells
were detected by immunohistochemical analysis of a subset of
gonads using polyclonal rabbit antibodies against Atlantic salmon
Vasa (Škugor et al., 2016) as primary antibodies and the ImmPRESS
polymerized reporter enzyme staining system (Vector) with horse-
radish peroxidase anti-rabbit immunoglobulin G as secondary anti-
body according to manufacturer’s protocol. Sections treated without
the primary antibody were used as negative controls.

5.3 | Sertoli cell number and tubuli size

Sertoli cells in the testicular tubuli of sterile and fertile testes were
counted in immunostained sections to avoid any misinterpretation of
cell types. Ten tubuli from 5 to 6 individuals of each male group
(immature sterile, mature sterile, immature fertile, and mature
fertile males) were used for calculating mean Sertoli cell numbers.
Tubular cross-sectional area was calculated according to the
formula πr² where the diameter (i.e., radius) was determined from
the average of two measurements of the same tubuli approxi-
mately perpendicular to each other. Mean cross-sectional area was
calculated from measurement of 10 different tubuli from 5 to 6
fish of each group.

5.4 | Sex steroid analyses

Plasma concentrations of 11-KT, testosterone (T), and oestradiol-17β
(E2) were quantified by means of radioimmunoassay (RIA), as
described by Schulz (1985). Assay characteristics and cross-
reactivities of E2 and T antisera have previously been examined
by Frantzen et al. (2004), and the 11-KT antiserum by Johnsen
et al. (2013). In short, nonconjugated steroids were extracted from
300 μl blood plasma with 4 ml diethyl ether under vigorous shaking
for 4 min. Subsequently, the aqueous phase was frozen in liquid
nitrogen and the organic phase was then transferred to a new glass
tube kept in a water bath at 45°C until all ether was evaporated.
Steroids were reconstituted by adding 900 μl of RIA-buffer and
then assayed for each of the sex steroids. A total of 79 individual
male plasma samples were analyzed for both T and 11-KT, whereas
66 female samples were analyzed for both T and E2, covering all
(with a very few exceptions) individuals subject to gene expression
analyses.
RNA extraction and complementary DNA synthesis

Total RNA was extracted from brain, pituitary, and gonads using conventional TRIzol method. Briefly, organs were homogenized in a TissueLyser (Qiagen) using steel beads with Trizol (Invitrogen). Homogenized samples were treated with chloroform, and RNA was precipitated with isopropanol. The RNA pellet was washed with 80% ethanol and dissolved in nuclease-free water. Genomic DNA contaminant was removed with DNase treatment using TURBO DNAfree TM Kit (Thermo Fisher Scientific) according to the manufacturer protocol. The quality and concentration of the RNA were determined spectrophotometrically by Nano Drop (Nano Drop Technologies). The measured A260/A280 ratio of 1.9–2.0 indicated high purity RNA.

RNA sample was reverse-transcribed with High-Capacity RNA-to-cDNA™ Kit (Thermo Fisher Scientific) using 200 ng RNA in 20 μl reaction volumes. Negative control without reverse transcriptase enzyme was used for examination of any genomic DNA contamination. The reaction was incubated in a thermocycler for 37°C for 60 min and stopped by heating at 95°C for 5 min before hold at 4°C.

The synthesized complementary DNA (cDNA) was diluted 1:40 and used as a template for qPCR analysis.

qPCR analysis

qPCR was used to measure relative expression of genes along the BPG axis. Specific primers were designed using Primer blast (NCBI) and Integrated DNA Technologies for amplification of 18 target genes and 3 reference genes (Table 2). The amplification efficiency of each primer pair was calculated using a twofold dilution series of a cDNA mixture according to the equation: 

\[ E = 10^{(-1/\text{slope})} \]  

(Pfaffl, 2001). The melting peak for each amplicon was inspected to check for unwanted amplification products. A control reaction to verify the absence of genomic DNA was conducted on three randomly selected RNA samples. The qPCR was run in duplicates in 7500HT sequence Detection system (Applied Biosystems) using the following recommended parameters: Standard run mode with 40 cycles at 50°C for 2 min, 95°C for 10 min, and 60°C for 1 min. Following by the melt curve stage at 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s. Cₜ threshold was set between 0.1 and 0.2. Each

### Table 2 qPCR primer sequences and amplification efficiency of the genes examined in Atlantic salmon

| Gene name  | Forward primer 5′-3′ sequence | Reverse primer 5′-3′ sequence | Efficiency |
|------------|-------------------------------|-------------------------------|------------|
| Dnd (JN712911) | CACAAGGGAGGGAGCAACTG | GCACAAGGGAGGGACACTG | 1.83 |
| Vasa (JN712912) | CCAGTACAGAAGCATGGCATTC | CCGTTTTCCAGATCCAGTCT | 1.92 |
| Igf3 (XM_014146080.1) | GACCGACCGACAAGATGCA | GCAAGGCACAATATGGAGTACA | 1.95 |
| Amh (NM_001123585.1) | CAGTCACCTCCTGAGCCTTACC | CAAACTTAATCCTTTCATTTAC | 1.82 |
| Cyp19a1a (XM_014175249) | TCAACAGAACCCCTGAGTAC | GCTCCCCCTACCTATAGCAGT | 2.19 |
| Cyp17a1 (XM_014154002.1) | TCCCCATGGCTACAGGTCTTC | CTGGTTTGGAGGACGAGGATT | 2.16 |
| Fshβ (XM_014126341.1) | TCACGGGACGCTACCCATTCA | GCTCTTGCAACCGGTATGA | 1.78 |
| LHβ (XM_014179761.1) | TACAGTGAGAGCCACGCTGA | CACAGTGCAAGAGGATTT | 2.1 |
| GnRH3a (XM_014206827.1) | GAGAGGCTGCTAGAGGCAT | ATGGTGATAGTGATGCTGAAA | 1.83 |
| GnRH3b (NM_001123667.1) | GAGAAGATATCTCCCAGAGCTCCTTA | TACAGGAGCTGAGGAGGAGGA | 1.76 |
| GnRH4a (MF073197.1) | ATATGAAGCCGACGTTAGA | TGAAGGTGTAGCGATGGT | 2.0 |
| GnRH4b (MF073197.1) | ATTTTTAGCTAGCTTCTT | TCTCATGCTAATGCTT | 1.96 |
| Fshr (NM_001123610.1) | CACAGGAGCCGCTGTGTA | GTGCCCTTGGCTTGCTGTA | 1.94 |
| 11βhsd2 (XM_045714152) | GCTGCTAGTGAGACCAGCGAG | GCCAGTGATGAGACAG | 1.89 |
| STAR (XM_014171084) | ATGGACCCCAAAGACCAAGA | GGGATCCAGGCTTTTAAATC | 1.86 |
| Cyp11b (XM_045699349) | GGAATGGCAGCTGCTGCT | AGGCTGAGGAGGAGGAG | 1.97 |
| Wnt5 (XM_014134623.1) | TAGCCAGGCTGCTGCTGCTTA | TGGGCTTTGGCTTGCTGCT | 1.96 |
| Ins13 (MF062497) | GTGACCCACACAGGTAAT | CTGCTTCTCGTCTCGT | 1.82 |
| EF1-a (AF321836) | CGCCAAATGGGCTCGTG | TCAACCATTGGCTTAC | 2.02 |
| β-actin (BT059604) | CAGCCCTCTTCTCTGCTTA | CGTCAACCTCTCTGCTGTTG | 2.03 |
| 18S (AJ427629) | TGTGCCGCTAGAGGGAATT | CGAACCCTTCAGCTTCTGC | 1.94 |

Abbreviation: qPCR, quantitative polymerase chain reaction.
well contained Fast SYBR Green PCR Master Mix, 500 nM final concentration of each primer, 5µl diluted cDNA (1:40) and nuclease free water (Ambion) to a final reaction volume of 15µl. All data were collected by the 7500 Software and Analysis Software (Applied Biosystem) and exported to Microsoft Excel for further analyses. The Pfaffl method was used to calculate relative expression (Pfaffl, 2001). The geometric mean (Vandesompele et al., 2002) of the three reference genes B1-actin, elongation factor 1α (ef1α), and ribosomal protein 18S were used to normalize the gene expression and remove nonbiological variation. Values from the control fish was used as calibrator as denoted by Pfaffl (2001).

5.7 | Statistics

All statistical analyses were performed with SYSTAT 13 software (SYSTAT 13 © 2009; SYSTAT Software Inc.). A Shapiro–Wilk test was used for testing normality and the Levene’s test was used for testing homoscedasticity. The quantile range method (Q = 1.5; tail 0.25) was used to identify outliers. Most data, except body weight and CF, were log transformed to meet test criteria. Differences in morphometric data, plasma sex steroid concentrations, and gene expression were analyzed for statistical significance by one- or two-way analysis of variance (ANOVA), followed by a Sidak multiple comparison test. Since maturing fish was first discovered at the last sampling point, only immature fish was subject to two-way ANOVA (time, fertility, and the interaction time × fertility). Normality for three of the plasma testosterone data sets was not obtained, and a nonparametric Kruskal–Wallis test followed with a Conover–Inman test for all pairwise comparison was used for this variable. For GSI and igf3 expression, homogeneity of variance was not possible to meet, and a Game–Howell multiple comparison test was used. Although log-transformed data was used for statistical evaluation, the original/actual data are presented in the figures as box plots. Weight and CF are presented as mean ± standard errors of mean. Complete datasets for all variables for each individual was not always possible to achieve, and number of fish within groups may, therefore, differ slightly between variables investigated.

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CONFLICT OF INTEREST
The authors declare no conflict of interest.
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SUPPORTING INFORMATION

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