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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 program and the steroidogenic function was maintained despite significantly reduced gonadotropic follicle-stimulating hormone (Fsh) levels (Maatouk et al., 2012; McNeilly 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 (Tak*ifugu alboplumbeus*), and salmonids, 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, Urano, 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 (C_t > 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).

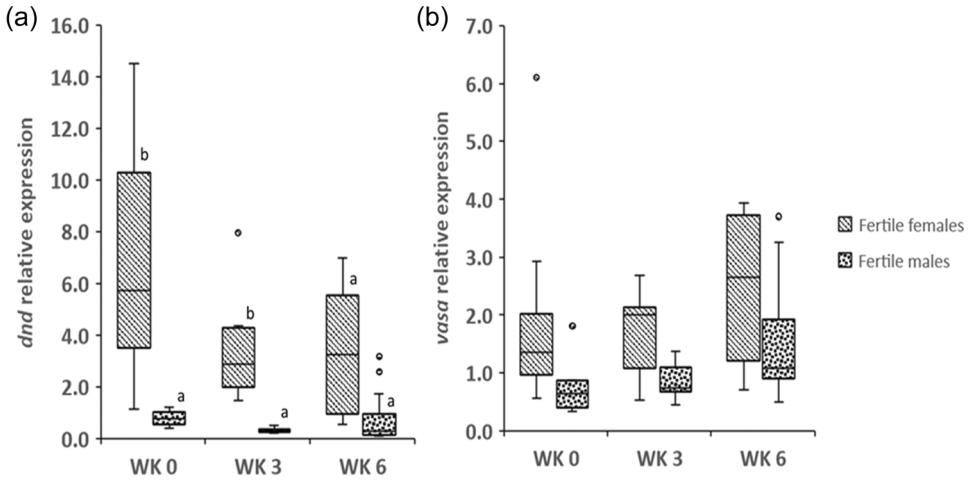


FIGURE 1 Temporal changes in gonadal relative expression of the germ cell marker genes *dnd* (a) and *vasa* (b) in fertile female ($n = 8–16$) and fertile male ($n = 9–16$) Atlantic salmon during exposure to continuous light for 0 (WK 0), 3 (WK 3), and 6 (WK 6) weeks at 10°C in freshwater. Before long 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.

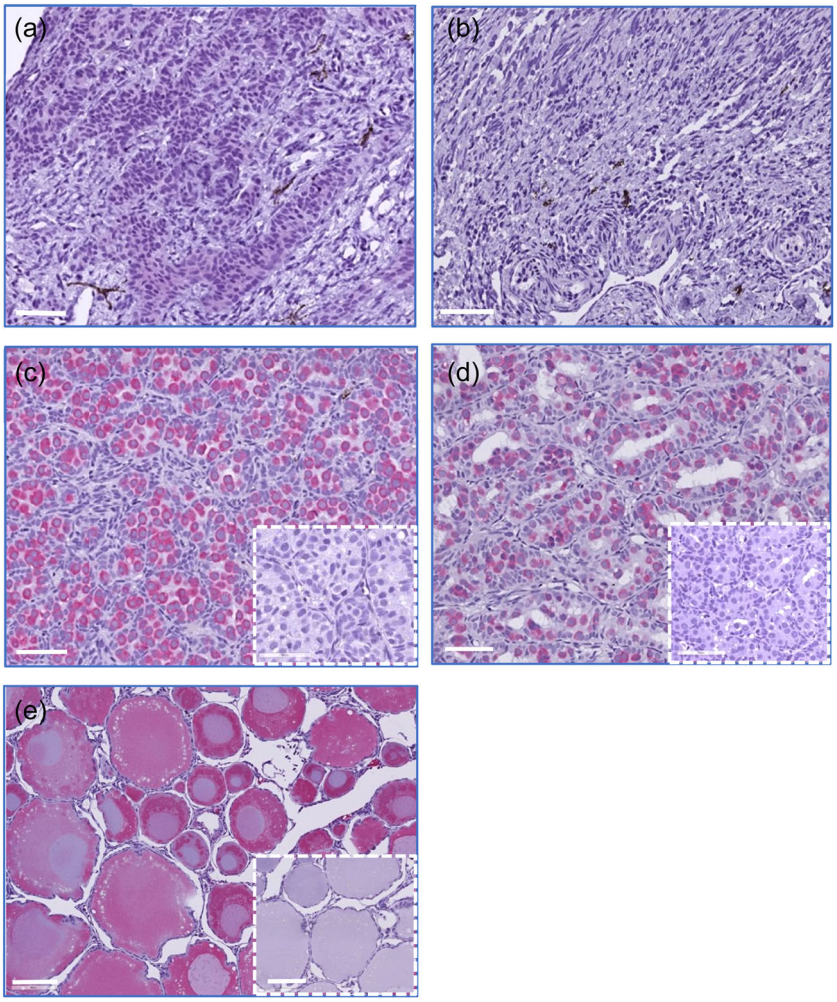


FIGURE 2 Immunohistochemical analysis of germ cell-specific Vasa (red staining) in Atlantic salmon gonads. (a) *dnd*-knockdown male, (b) *dnd*-knockdown female, (c) fertile male with undifferentiated spermatogonia, (d) fertile male with differentiated spermatogonia, and (e) fertile female with previtellogenic oocytes as the most advanced stage of development. Negative controls are inserted. Scale bar = 60 µm.

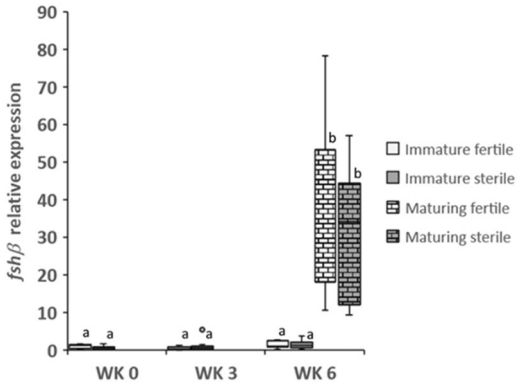


FIGURE 3 Temporal changes in pituitary *fshβ* relative gene expression in immature fertile (□ $n = 6–13$), immature sterile (■ $n = 8–11$), maturing fertile (▣ $n = 9$), and maturing sterile (▣ $n = 9$) male Atlantic salmon during exposure to continues light for 0 (WK 0), 3 (WK 3), and 6 (WK 6) weeks 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.

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 3 weeks (WK 3) after exposure to long day (Figure 3). However, 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 >*c*.10, $n = 9$, or 60%) were evaluated as having initiated maturation, while those in the lower mode (*fshβ* relative expression <*c*.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 *lhβ* 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

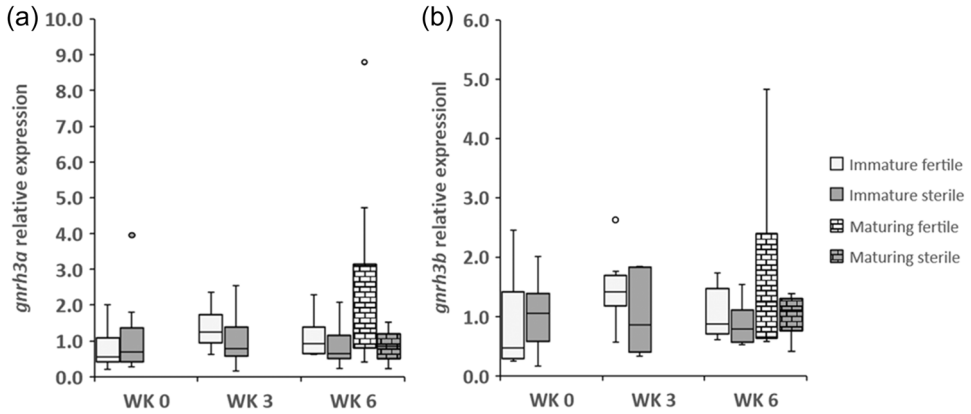


FIGURE 4 Temporal changes in brain *gnrh3a* (a) and *gnrh3b* (b) relative gene expression in immature fertile (n = 6–12), immature sterile (n = 8–12), maturing fertile (n = 9), and maturing sterile (n = 8–9) male Atlantic salmon during exposure to continues light for 0 (WK 0), 3 (WK 3), and 6 (WK 6) weeks at 10°C in freshwater. *See* Figure 1 *for more details*.

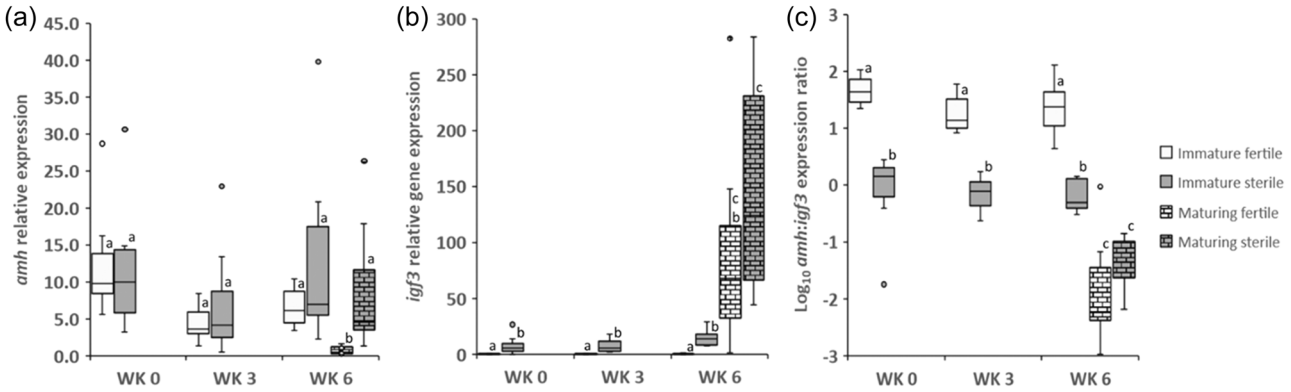
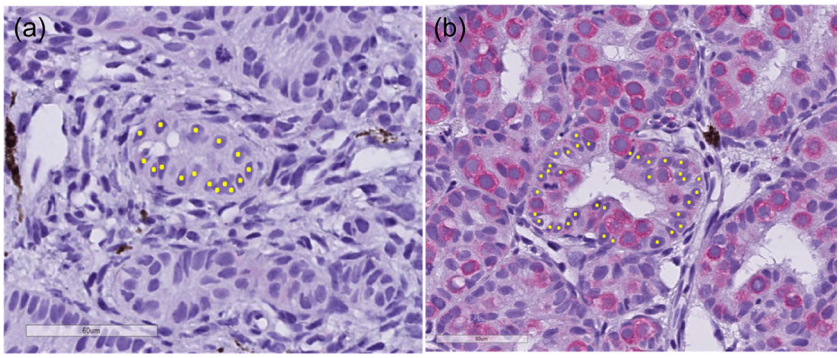


FIGURE 5 Temporal changes in testicular *amh* (a), *igf3* (b), and *amh:igf3* ratio ($\log_{10}$ values) (c) relative gene expression in immature fertile (n = 6–13), immature sterile (n = 8–13), maturing fertile (n = 9), and maturing sterile (n = 9) male Atlantic salmon during exposure to continues light 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.

FIGURE 6 Sertoli cells in cross-sectioned testicular tubuli of maturing sterile (a) and fertile (b) Atlantic salmon males. Sertoli cells are indicated by yellow dots whereas germ cells are indicated by positive staining (red) for Vasa protein. Scale bar = 60 µm.



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 gr 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).

TABLE 1 Number of Sertoli cells and cross-sectional area of seminiferous tubuli in immature and maturing sterile and fertile Atlantic salmon males

Male subgroups	Sertoli cell number	Tubuli cross-sectional area (mm^2)
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.

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

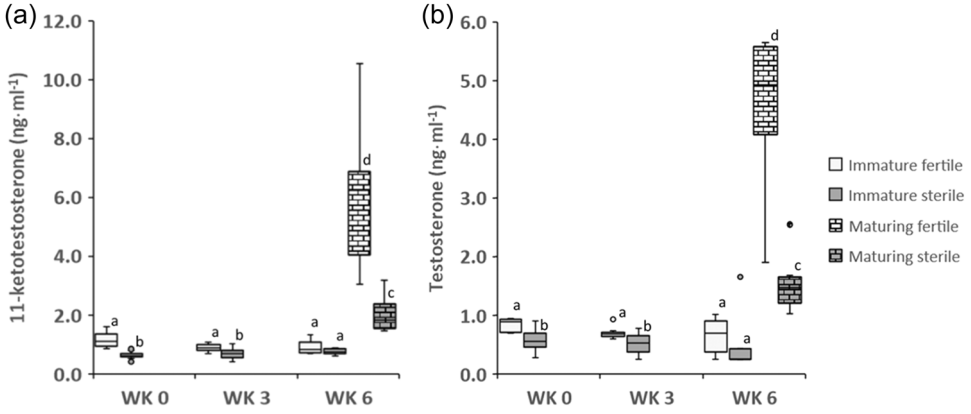


FIGURE 7 Temporal changes in 11-ketotestosterone (a) and testosterone (b) plasma concentrations ($ng \cdot ml^{-1}$) in immature fertile ($n = 5–12$), immature sterile ($n = 7–13$), maturing fertile ($n = 9$), and maturing sterile ($n = 8$) male Atlantic salmon during exposure to continues light for 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.

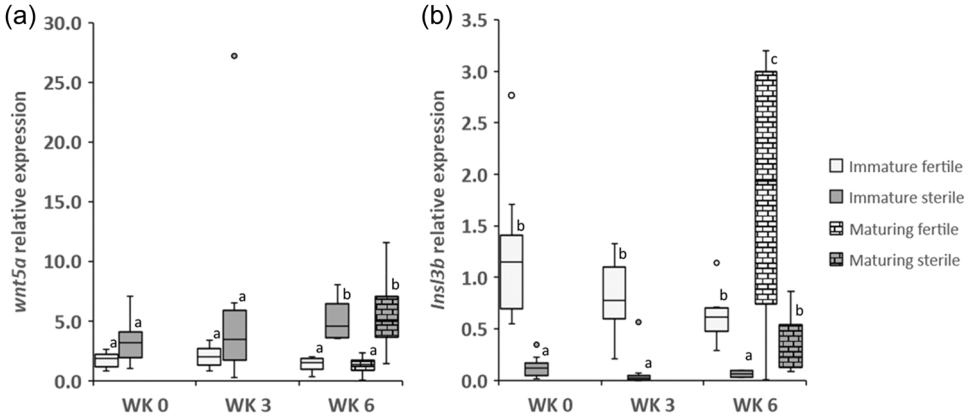


FIGURE 8 Temporal changes in testicular *wnt5* (a) and *insl3b* (b) relative gene expression in immature fertile (□ $n = 6–13$), immature sterile (■ $n = 8–13$), maturing fertile (▤ $n = 9$), and maturing sterile (▦ $n = 9$) male Atlantic salmon during exposure to continues light for 0 (WK 0), 3 (WK 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.

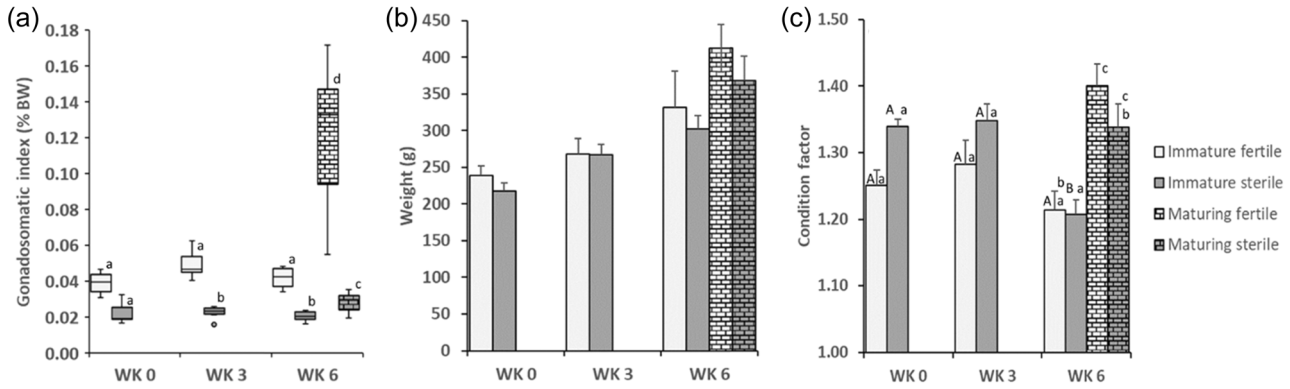


FIGURE 9 Temporal changes in gonadosomatic index (GSI) (a), body weight (b), and condition factor (CF) (c) in immature fertile (□ $n = 5–13$), immature sterile (■ $n = 8–13$), maturing fertile (▤ $n = 9$), and maturing sterile (▦ $n = 9$) male Atlantic salmon during exposure to continues light for 0 (WK 0), 3 (WK 3), and 6 (WK 6) weeks at 10°C in freshwater. GSI and CF are presented as means ± standard errors of mean. 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 Insl3 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 Insl3 (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 spermatogonial 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 testis of this stage (Güralp 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 masu 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* (*gnrhr2bbα*) was moderately upregulated concomitant with increased *fshβ* levels in maturing salmon parr, but was exclusively localized in *lhβ* expression cells (Ciania 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 *fshb* expression occurred 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) (Aksnes 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 Kårvika, 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 undertaken 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 microinjected 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 RNAase-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 according to prevailing temperatures, water O_2 saturation >80% and fish densities of *c.* 25 kg/m^3) with continuous light (24L:00D) at 6–10°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), smoltification was induced by exposing the fish to a photoperiod of daily 6 h light (6L:18D) and water temperature of 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), anaesthetized 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)^3)$^{-1}$. 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 bisected with one part fixed in PFA for histology and immunohistochemistry, 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 and females (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 horseradish peroxidase anti-rabbit immunoglobulin G as secondary antibody 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 $\pi \cdot r^2$ where the diameter (i.e., radius) was determined from the average of two measurements of the same tubuli approximately 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.

5.5 | 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.

5.6 | 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 Dection 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. C_t 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)	CACAAGGAGGGAGCAACTG	GCACAAGGAGGGAGCAACTG	1.83
Vasa (JN712912)	CCAGTACAGAAGCATGGCATTC	CCGTTTTCCCAGATCCAGTCT	1.92
Igf3 (XM_014146080.1)	GACCGACCGACAAGATGCA	GCAAGGCACAATATGGAGTACA	1.95
Amh (NM_001123585.1)	CAGTCACTCTCTGCAGCCTTACAA	CAACATTGAATCTCCATTTCAGTTTAC	1.82
Cyp19a1a (XM_014175249)	TCAAACAGAACCCTGACGTAG	GCTCCCTTTCACCTATAGCAGTGT	2.19
Cyp17a1 (XM_014154002.1)	TCCCATGGCTACAGGTCTTC	CTGCTTTAGGAGACGCAGGT	2.16
Fshβ (XM_014126341.1)	TCACGGAGGCATCACCATCA	GCTCTTGGCAACGGGTATGA	1.78
LHβ (XM_014179976.1)	TACAGTGAGCACGCCATCGA	CCAGCTGCAAGGCATGAGTT	2.1
GnRH3a (XM_014206827.1)	GAGAGGCTGAGACCATAT	ATGTTGATAGTGATGCTGAA	1.83
GnRH3b (NM_001123667.1)	GAGAAAGTATCTCCCATGGATCTTA	TACGACCAGTGCTGAGAGAGA	1.76
GnRHR4a (KF225730.1)	ATATGAAGCAGGCGTTAGA	TGAAGTTGTAGCAGATGGT	2.0
GnRHR4b (MF073197.1)	ATTGTTATTGTGACTTCCTT	TCTCATCCATATCGTCTG	1.96
Fshr (NM_001123610.1)	CCACGGGACGCTGTGTTAAC	GTGTCCCATGCCTGGTCTTG	1.94
11βhsd2 (XM_045714152)	GCTGCCTATACTCTGCCA	GCCTGTGATGAAGACAGC	1.89
STAR (XM_014171084)	ATGACCCCAACAAGACCAAG	GGGATCCAGCCCTTTAAATC	1.86
Cyp11b (XM_045699349)	CGAAATGCAGCTGCTACTGA	AGGCTGGAGGATTAGGGTGT	1.97
Wnt5 (XM_014134623.1)	TAGGCAGGCTGTGCAATAAG	TGGGCTTTGTACTGGTCATATC	1.96
Insl3 (MF062497)	GTCAACCACCACAGGGTAAT	CTGGTCTTCTGTCATGCTCTC	1.82
EF1-α (AF321836)	CGCCAACATGGGCTGG	TCACACCATTGGCGTTACCA	2.02
β-actin (BT059604)	CAGCCCTCCTTCCTCGGTAT	CGTCACACTTCATGATGGAGTTG	2.03
18S (AJ427629)	TGTGCCGCTAGAGGTGAAATT	CGAACCTCCGACTTTCGTTCT	1.94

Abbreviation: qPCR, quantitativepolymerase chain reaction.

well contained Fast SYBR Green PCR Master Mix, 500 nM final concentration of each primer, 5 µl diluted cDNA (1:40) and nuclease free water (Ambion) to a final reaction volume of 15 µl. 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 *β1-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

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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