Sex reversal and sex differentiation in Atlantic Salmon (Salmo salar)

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Executive Summary

Background

The Tasmanian Atlantic Salmon (*Salmo salar*) aquaculture industry strives to produce all-female fish, as male Atlantic Salmon are subject to precocious maturation and consequently reduced flesh quality and increased disease susceptibility when reared in high water temperatures. Several fish species, including Atlantic Salmon, can have their natural sex ratio changed by sex reversal, which involves the application of steroid (androgens and estrogens) and non-steroid (aromatase inhibitors) substances (by immersion or inclusion in the diet) during early stages of development, most of the time prior to phenotypic (morphological) sex differentiation. The timing of application of these substances is instrumental for achieving high rates of sex change. All-female populations can be produced by crossing a masculinized female (XX male or neomale) with a normal female (XX female). Therefore, it is important to test the potential of different substances in producing neomales, which then can be crossed with normal females to consistently produce all-female fish. This indirect method leads to the production of individuals that were never exposed to exogenous steroids.

Aims/objectives

The overall aim of this project was to integrate farm-based trials, histology, and genetic technologies to evaluate options for the improved production of all-female Atlantic Salmon in Tasmania. More specifically, we:

1. Investigated morphological sex differentiation processes in male and female Atlantic Salmon.
2. Quantified the expression of several key genes involved in sex differentiation in male and female Atlantic Salmon.
3. Investigated the effects of 17a-methyldihydrotestosterone (MDHT) treatment on expression of several key genes involved in sex differentiation in female Atlantic Salmon.
4. Investigate the effects of dimethyl sulfoxide (DMSO) on the masculinizing potential of a single MDHT immersion treatment in female Atlantic Salmon.
5. Investigated the effects of the aromatase inhibitor letrozole applied at different developmental stages prior to first feeding on masculinization of all-female population of Atlantic Salmon.

Methodology

We used a combination of morphological (histology, visual inspection, biometry), genetic, and gene expression techniques to address the questions above. For the sex differentiation studies, a mixed sex population of Atlantic Salmon was used, and their genetic sex, gonadal morphology and gene expression were examined during gonadal sex differentiation. For the sex reversal studies we used an all-female population (obtained via the cross of a neomale with a normal female) and subjected these individuals to immersion treatments in an androgen (MDHT) alone or in combination with a solubilizing agent (DMSO), and an aromatase enzyme inhibitor (letrozole) that prevents the conversion of male hormones (androgens) to female hormones (estrogens). Gonadal morphology, sex proportion and gene expression (MDHT study only) were determined at different days after treatment.

Results/key findings
The histological observations of gonad development and sex differentiation in Atlantic Salmon (objective 1) revealed that phenotypic sex can only be clearly distinguished at 79 days post hatching (DPH). Based on one of the histological criteria used (arrangement of germ cells in well-defined cysts), differentiation of ovary was becoming evident at 52 DPH. At 79 DPH, the genetic sex of all examined fish corresponded 100% to their morphological (phenotypic) sex.

The expression of sex-related gonadal genes (objective 2) demonstrated that the only genes exclusively present in males were *amh* and *sdY*, and in females *cyp19a1a*. Expression of *gsdf* and *foxl2* was detected in males and females. We also determined the expression of a sex-related neural gene (*cyp19a1b*), and 4 retinoic acid genes (*aldh1a2*, *cyp26a1*, *cyp26b1* and *tbx1a*) and there was no difference between the sexes.

Immersion treatment of an all-female population of Atlantic Salmon in MDHT (objective 3) led to the upregulation of *amh* and *gsdf* (male specific genes), and down regulation of *cyp19a1a* (female specific gene) at 56 days after treatment (75 days post hatching).

Immersion treatment in DMSO + MDHT (objective 4) or MDHT only increased the proportion of males to 87% and 81%, respectively. In these groups the proportion of females was 3% (DMSO + MDHT) and 9% (MDHT). In both groups approximately 6% of the individuals were intersex (presence of both gonads) and 3% sterile. The proportion of males in the control and DMSO only groups was 6 and 0%, respectively. Contrasting with the MDHT results, immersion of an all-female population of Atlantic Salmon in letrozole (a non-steroidal masculinizing agent) (objective 5) at different times during the sex differentiation period did not cause masculinization.

Our studies are indicating that:

1. Based on histology and morphology, sex differentiation in Atlantic Salmon seems to occur by 79 dph.
2. Based on gene expression, three male specific genes are upregulated prior to morphological sex differentiation, whereas in females only one gene is differentially upregulated prior to morphological sex differentiation.
3. Immersion treatment with MDHT and MDHT+DMSO led to masculinization of genetic females to rates between 81 and 87%.
4. Immersion in MDHT led to the upregulation of male specific genes, which occurred 15 days after (dph) the date observed in normal males (34-66 dph) in the sex differentiation study.

Implication for relevant stakeholders
The implications of these findings are relevant to the industry and academic/research communities as they demonstrate that is possible to obtain higher rates of masculinization using androgens but not aromatase inhibitors. The higher rates of masculinization will provide more neomales that can be grown to maturity and crossed with normal females to produce all-female populations of Atlantic Salmon. The combination of MDHT + DMSO increased the rate of masculinization (although not significantly different from MDHT alone), and further studies in this area could lead to the optimization of a sex reversal protocol to further increase the rates of masculinization. The sex differentiation and gene expression studies are showing that Atlantic Salmon is a unique species regarding these aspects, and this information needs to be taken into consideration when developing sex reversal protocols.

Recommendations
Further studies in the areas of sex differentiation and sex reversal are necessary in order to maximize the sex reversal protocols for the production of neomales. In addition, it will be important to follow up in long-term studies the effects of these masculinizing protocols in juvenile and adult fish. For example, 1: can the sex reversal be only temporary, and neomales could eventually revert and develop as females? 2: will the crosses of neomales with normal females lead to 100% females. Thus, examination (histology

and gene expression) of F1 individuals in a long-term (possibly longitudinal studies) should help us to answer those questions.

Keywords

Atlantic Salmon, gene expression sex differentiation, sex reversal

Introduction

The overall aim of this project was to integrate farm-based trials, histology and genetic technologies to evaluate options for the improved production of all-female Atlantic Salmon (*Salmo salar*).
Most fish species, including Atlantic Salmon, are quite susceptible to sex manipulation during early stages of development. This characteristic can be manipulated in the hatchery environment to produce mono-sex populations. All-female Atlantic Salmon are produced commercially in Tasmania, as males are more likely to undergo maturation prior to harvest size which results in reduced flesh quality and increased disease susceptibility (Aksnes et al., 1986; Elliott and Kube, 2009; Piferrer et al., 2012).

One approach to produce monosex populations is through sex reversal, whereby steroid or non-steroid substances are applied (by immersion or diet) to fish in the hatchery at early stages of development. For example, androgens are potent masculinising agents in fish. Several studies have demonstrated that treatment with exogenous androgen (synthetic products such as methyltestosterone and 17α-methyldihydrotestosterone) can result in 100% male populations. One of the issues of using androgens for sex reversal is public perception, which is related to the fact there could be residuals of the substance in the fish muscle. Another approach that overcomes the public perception issue, is indirect sex-reversal, whereby ovum from normal female brood stock (genetic sex XX) are fertilised by sperm from masculinised females, termed neo-males (also XX) to produce all female offspring. However, current commercial methods for masculinising genetically female Atlantic Salmon, which have been developed from research conducted ~15 years ago at SALTAS (Lee et al., 2004), utilise steroidal androgens which contribute to negative public perception of the Atlantic Salmon industry.

One of the crucial aspects to the success of any sex reversal protocol is the timing when the androgens are applied. This is because sex reversal treatments are most effective when applied just prior or simultaneously with the initiation of sex differentiation (Hunter and Donaldson, 1983; Navarro-Martín et al., 2009). Sex differentiation refers to conserved physiological pathways that mediate phenotypic divergence of gonadal germ cells into respective ovarian or testicular tissues (Piferrer and Guiguen, 2008). Traditionally, sex differentiation has been investigated using histology, to discern morphological processes within gonadal tissue. However, it has since been demonstrated that the expression of genes encoding proteins involved in sex differentiation are initiated prior to morphological differentiation processes (Piferrer and Guiguen, 2008). Ovarian differentiation is characterised by early expression of genes which function in estrogen biosynthesis and promote ovarian development (Baron et al., 2005; Vizziano et al., 2007; Ijiri et al., 2008; Guiguen et al., 2010). Testicular differentiation is marked by early upregulation of genes which antagonise ovarian pathways and promote androgen production (Vizziano et al., 2008).

The current project is needed because the Australian Atlantic Salmon Industry is lacking basic and applied knowledge that could lead to the development of a reliable, non-steroidal method to produce all-female populations of Atlantic Salmon. One of the drivers of this project is the fact that eliminating males from the production cycle (grow-out period), will reduce the effects of precocious sexual maturation, including reduced growth, reduced flesh quality, and susceptibility to diseases. Consequently, eliminating males from the production cycle will increase overall productivity (biomass produced) and profitability.

The use of modern genetic and morphological techniques to improve our understanding of sex differentiation in Atlantic Salmon is needed to inform the exact period that sex reversal treatments with exogenous substances are most effective for producing neo-males. This exact timing can then be used in the trial of next generation non-steroidal substances, ensuring that they have the greatest chance of success. This will be the first time that detailed gene expression and morphological information will be collected throughout the entire period of sexual differentiation in Atlantic Salmon.

Objectives

The objectives of this project were:

1. Investigate morphological sex differentiation processes in male and female Atlantic Salmon larvae.
2. Quantify the expression of several key genes involved in sex differentiation in male and female Atlantic Salmon embryo's and male and female Atlantic Salmon larvae (gonadal and estrogenic non-gonadal tissues).
3. Investigate the effects of MDHT treatment on expression of several key genes involved in sex differentiation in female Atlantic Salmon larvae (gonadal and estrogenic non-gonadal tissues).
4. Investigate the effects of DMSO on the masculinizing potential of a single MDHT immersion treatment in female Atlantic Salmon.
5. Investigate the effects of the aromatase inhibitor letrozole on expression of several key genes involved in sex differentiation in female Atlantic Salmon larvae (gonadal and estrogenic non-gonadal tissues).

Methods

Three studies were carried out at SALTAS (Tasmania, Australia).

1. Sex differentiation study (2018)

1.1. Experimental design

This was a four month experiment, which required collection of Atlantic Salmon embryos (at 3, 11, 29 and 46 days after fertilisation) and larvae (at 0, 6, 12, 18, 26, 34, 44, 52, 59, 66, 72 and 79 days after hatching) from a mixed sex population. The fish used in this study were maintained under commercial conditions. Prior to complete yolk sac absorption, embryos and alevins used in this experiment were reared at 8.27°C ± .04. Once ponded, fry were reared at 10.77°C ± 0.21 until termination of the experiment. Median day of hatch and yolk sac absorption occurred at 468 degree days post fertilisation (ddpf) and 56 dph (928 ddpf), respectively. Once capable of independent feeding, Atlantic Salmon larvae were fed a high quality extruded pellet diet (Skretting Australia) *ad libitum* throughout daylight periods.

1.2. Sampling procedure

There were 3 replicate tanks in this study. At each sampling time, embryos and larvae were quickly captured and immediately placed in a lethal dose of anaesthetic (AQUI-S 30 mg L^{-1}). Embryos and larvae were collected for histology and gene expression (real-time qPCR) analysis. Histology was used to characterise morphological sex differentiation (Fatima et al., 2012), whereas qPCR was used to investigate gene expression during sex differentiation. For histology, 10 larvae were collected from each of the 3 replicate tanks at each of the 12 time points. Whole larvae were fixed in Bouin's solution. For gene expression analysis, 30 embryos or 20 larvae were collected from each of the 3 replicates at each of the 16 time points and placed in individual micro-centrifuge tubes filled with RNA*later*, then frozen at -80 °C.

1.3. Sample analysis

In addition to histology, expression of the following genes was investigated using real-time qPCR:

- sex differentiation-related genes: *amh* (*anti-Mullerian hormone*), *cyp19a1a* (*cytochrome P450, family 19, subfamily A, polypeptide 1a*), *foxl2a* (*forkhead box L2a*), *gsdf* (*gonadal soma derived factor*), *rspo1* (*r-spondin 1*) and *sdY* (*sexually dimorphic on the Y chromosome*)
- neural genes: *cyp19a1b* (*cytochrome P450, family 19, subfamily A, polypeptide 1b*)
- retinoic acid-signalling genes: *aldh1a2* (*aldehyde dehydrogenase 1 family, member A2*), *cyp26a1* (*cytochrome P450 Family 26 Subfamily A Member 1*), *cyp26b1* (*cytochrome P450 family 26 subfamily B member 1*), *tbx1a* (*T-Box Transcription Factor 1*)

2. Sex reversal studies

2.1 MDHT + DMSO (2018)

2.1.1. Experimental design

In this study, all-female Atlantic Salmon larvae were subjected to a single 2-hour immersion in one of the following 4 treatments; Control (DMSO-0 mg L^{-1} + MDHT-0 µg L^{-1}), DMSO-300 mg L^{-1}, MDHT-400 µg L^{-1} or a combination of DMSO and MDHT (DMSO-300 mg L^{-1} + MDHT-400 µg L^{-1}) at 19 days after hatch. There were 3 replicate tanks for each treatment. MDHT (≥97.0%) and DMSO (≥99.9%) were purchased from Sigma-Aldrich (catalogue no. M5626-5G and 276855-1L, respectively). This experiment required collection of all-female Atlantic Salmon larvae at 0 (prior to the immersion), 1, 3, 7, 16, 33, 56, 81 and 240-241 days after treatment. Prior to complete yolk sac absorption, embryos and alevins were reared at 8.12°C ± 0.48. After the onset of free swimming, fry were reared at 11.48°C ± 0.88 until 100 dph (1464 ddpf). Following, they were transferred to a flow-through aquaculture system and maintained in ambient water temperature for 54 days, then returned to the RAS and kept at ~15°C for the remaining duration of the experiment. Hatching and yolk sac absorption occurred at 475 ddpf and 52 dph (903 ddpf), respectively.

2.1.2. Sampling procedure

At each sampling time, 10 fish were sampled from each replicate tank of each treatment. Larvae collected for gene expression analysis were immersed in RNA*later* and stored at -80°C. The gonads of fish collected for histology were dissected out, immersed in Bouin's solution, and stored at room temperature.

2.1.3. Sample analysis

Histology was used to determine gonadal sex and therefore the efficacy of each treatment group for producing neo-males. Gene expression analysis (real-time qPCR) was used to investigate the effects of MDHT on the expression of the sex differentiation-related genes listed above.

2.2 Aromatase Inhibitor Letrozole (2019)

2.2.1. Experimental design

In this study, all-female Atlantic Salmon larvae were subjected to two 6-hour immersions in letrozole (10 mg L^{-1}; applied 8 days apart) initiated at either 7 days before hatching, 1 day after hatching or 9 days after hatching. There were 3 replicate tanks for each treatment. Letrozole (≥98.0%) was purchased from Sigma-Aldrich (catalogue no. L6545-50MG). Prior to complete yolk sac absorption, embryos and alevins were reared at 8.06°C ± 0.37. After the onset of free swimming, fry were reared at 14.64°C ± 0.98 for the duration of the experiment. Hatching and yolk sac absorption occurred at 466 ddpf and 55 dph (915 ddpf), respectively.

2.2.2. Sampling procedure

At each sampling time, 10 fish were sampled from each replicate tank of each treatment. Larvae collected for gene expression analysis were immersed in RNA*later* and stored at -80°C. The gonads of fish collected for histology were dissected out, immersed in Bouin's solution, and stored at room temperature.

2.2.3. Sample analysis

Gross observation of gonadal morphology was used to determine gonadal sex and therefore the efficacy of each treatment group for producing neo-males.

3. Statistical Analysis

All statistical analysis was performed with R 3.6.1 (www.r-project.org). mRNA expression data from both experiments as well as length and histological data from the sex differentiation experiment were analysed using the nlme package 3.1-140. A linear mixed-effects model (LME, (Pinheiro et al., 2017)) was used to account for differences between tanks that were measured repeatedly over time. Treatment (sex reversal experiment) or sex (sex differentiation experiment) and time (and the interaction) effects were tested, by specifying treatment/sex and time as fixed effects and tank identity as a random (intercept) effect.

Treatment/sex and time were modelled as categorical variables. Planned post hoc comparisons were used to investigate significant interactions and main effects, with a false discovery rate (FDR) used to account for multiple comparisons (Benjamini and Hochberg, 1995). For the sex reversal experiment, treatment effects

on survival, length, and mass at 81 days after treatment (dat) were investigated using a one-way ANOVA (AOV). For both LME and AOV analyses, the assumption of homogeneity of variance was checked by examining the residual plotted against the fitted values and using a Levene's test on the residuals. The assumption of normal distribution was checked using a quantile-quantile plot and Shapiro Wilk test on the residuals. Where necessary, transformations were used to improve heteroskedastic variance and non-normal distributions.

For the sex reversal experiment, a chi-square (χ^2) test was used to determine the effect of treatment on sex ratio at 81 and 240-241 dat. In this analysis, 'undifferentiated' fish observed at 81 dat were categorised with 'males' (Olito & Brock 1991), whereas 'intersex' and 'sterile' fish observed at 240 and 241 dat were categorised with 'females. Therefore, only females that were completely sex-reversed into males were considered to be affected by treatment (Baker et al., 1988; Piferrer and Donaldson, 1989; Piferrer and Donaldson, 1991; Feist et al., 1995). Multiple post hoc χ^2 tests were used when a significant effect of treatment on sex ratio was found (Kim, 2017). All data is presented as means ± S.E. and statistical significance was considered at a level of $p < 0.05$.

Results

Sex differentiation experiment

Histology

Histological examination of gonadal development in Atlantic Salmon revealed that at 18 dph paired gonads were observed in 100% of the collected samples (Fig. 1a). The gonads were located ventrally to the kidney and dorsally to the digestive tract. For most samples, the expected longitudinal position of the gonads could not be observed. However, the anterior end of the gonad was proximal to or immediately posterior of the pectoral fin joint in those samples that it could be seen. Gonad cross sections were predominantly oval shaped. Lumina were generally observed throughout the gonad cross sections. For most samples, germ cells were observed to be singularly dispersed in the distal portion of the gonad cross section. However, in some samples (4 out of 12), germ cells occasionally appeared to be clustered in a cyst-like formation. Phenotypic sex was not obvious in any of the samples analysed at this age.

At 34 dph, gonads were located directly ventral to the kidney in all samples (Fig. 1b). In 7 out of 12 samples, the gonads were in a clearly defined coelomic cavity. The anterior end of the gonads was proximal to the posterior end of the pectoral fin joint, or immediately posterior of it. Gonad cross sections were oval shaped. Lumina were not visible in most samples. Germ cells were mainly singularly dispersed in the distal portion of the gonad cross section. Phenotypic sex was not obvious in any of the samples analysed at this age.

At 52 dph, gonads were located directly ventral to the kidney, and within a clearly defined coelomic cavity in 11 out of 12 samples (Fig. 1c and d). The anterior end of the gonads was immediately posterior of the pectoral fin joint in all samples. In 9 out of the 12 samples, phenotypic sex was not obvious (Fig. 1c). In these samples, germ cells were mainly singularly dispersed throughout the middle and distal portions of the gonad cross sections. However, in 4 out of 9 of these samples, germ cells were also occasionally clustered in a cyst-like formation. Ovarian phenotype, as defined by arrangement of germ cells in well-defined cysts throughout the entire length of the gonad cross sections, was evident in 3 out of 12 samples (Fig. 1d). Independent of phenotype, gonad cross section shape varied from oval to triangular in all samples, and lumina were not observed.

At 66 dph, gonads were located directly ventral to the kidney within the coelomic cavity in all samples (Fig's. 1e and 1f). The anterior end of the gonads was immediately posterior of the pectoral fin joint in all samples. In 6 out of 12 samples, phenotypic sex was not obvious (Fig. 1e). In these samples, germ cells were mainly singularly dispersed in the distal portion of the gonad cross section. However, in 3 out of 6 of these samples, germ cells were also occasionally arranged in small cysts. In 2 out of these 6 samples, lumina and a vein were evident in the proximal region of the gonad cross sections. Ovarian phenotype, as defined by arrangement of germ cells in well-defined cysts throughout the entire length of the gonad cross sections, was evident in 6 out of 12 samples (Fig. 1f). In 3 out of these 6 samples, lumina were evident. In 2 out of these 6 samples, a vein was evident in the gonad. The position of the lumina and vein were variable. Independent of phenotype, gonad cross sections were mostly oval shaped.

At 79 dph, gonads were located directly ventral to the kidney, within the coelomic cavity in all samples (Fig's. 1g and 1h). The anterior end of the gonads was immediately posterior of the pectoral fin joint in all samples. In 7 out of 12 samples, phenotypic sex was unknown (Fig. 1g). In these 7 samples, germ cells were singularly dispersed in the middle and distal portions of the gonad cross section. All these 7 samples were demonstrated to possess *sdY* in gDNA, indicating they were genotypic males. In 2 out of 7 of these samples, germ cells were also occasionally arranged in small cysts. Generally, an artery, vein and/or lumen were evident in the proximal region of the gonad. Ovarian phenotype, as defined by arrangement of germ cells in well-defined cysts throughout the entire length of the gonad cross sections, was evident in 5 out of the12 samples (Fig. 1h). In 1 out of these 5 samples, early formation of an ovarian canal was also evident. All 5 of these samples were shown to lack *sdY* in gDNA, indicating they were genotypic females. An artery and vein were not well defined in the gonads of these samples, but lumina were evident in various locations within the cross section. Independent of phenotype, gonad cross sections were oval or triangular shaped.

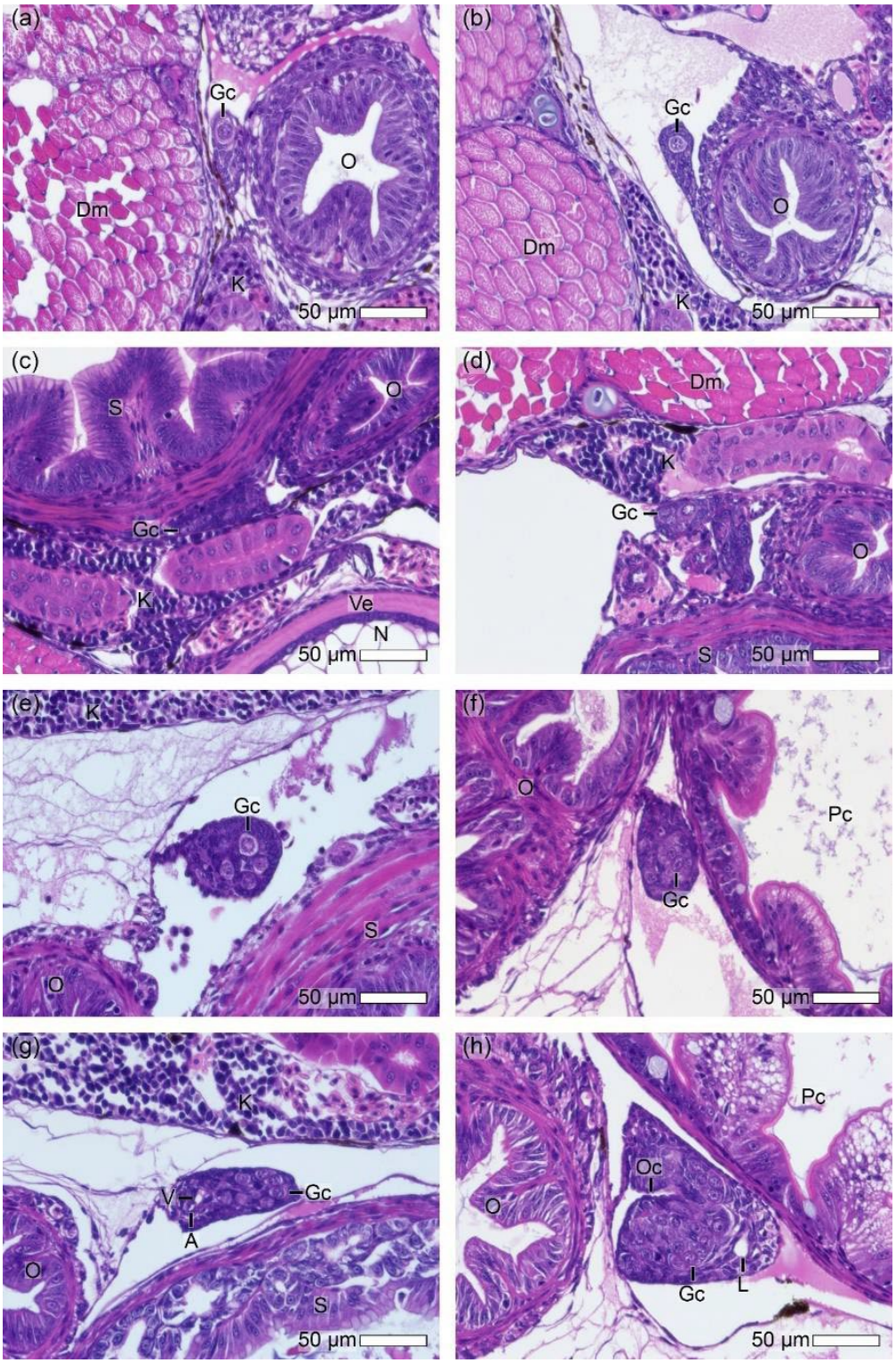

Figure 1. Histological assessment of gonad development and sex differentiation in a mixed-sex Atlantic Salmon population. (a) gonad with unknown phenotype at 18 days post hatch (dph) (12/12); (b) gonad with unknown phenotype at 34 dph (12/12); (c) gonad with unknown phenotype at 52 dph (9/12); (d) gonad with ovarian phenotype at 52 dph (3/12); (e) gonad with unknown phenotype at 66 dph (6/12); (f) gonad with ovarian phenotype at 66 dph (6/12); (g) gonad with unknown phenotype at 79 dph (7/12); (h) gonad with ovarian phenotype at 79 dph (5/12). Gc, germ cell; Dm, dorsal muscle; K, kidney; O, oesophagus; Ve, vertebra; N, notochord; S, stomach; Pc, pyloric caeca; V, vein; A, artery; Oc, ovarian canal; L, lumina.

Irrespective of phenotype, gonad cross section area increased from 52 dph (Fig. 2a). There were no significant changes in germ cell number (Fig. 2b) or germ cell diameter (Fig. 2c) during this period. No significant phenotype-related differences in gonad cross section area, germ cell number or germ cell diameter were evident during the experimental period.

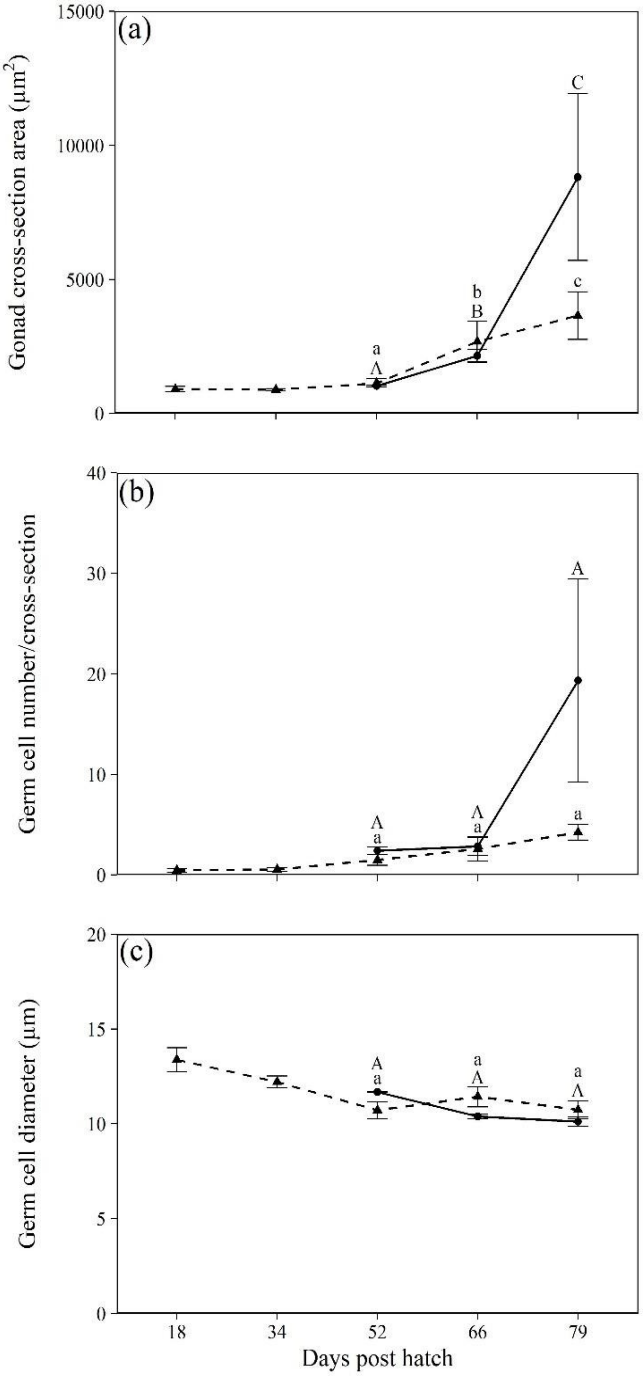

Figure 2. Mean (± S.E.M.) gonad cross-section area (a), germ cell diameter (b) and germ cell number in Atlantic Salmon with unknown -▲- and female -●- phenotype at various ages during sex differentiation. Significant differences ($p < 0.05$) within the unknown group are indicated by lowercase letters, and within the female group ($p < 0.05$) by different uppercase letters. * indicates significant differences between phenotype groups within time ($p < 0.05$).

Genetic determination of sex

The *sdY* copy number was used to determine the genotypic sex of each sample required for gene expression studies (Fig. 3). After quantification of the sdY copy number, samples were separated by sex. Fish with >2500 copies were designated as males, and fish with 0 copies were designated as females. Fish samples with sdY copy number higher than zero but lower than 2500 copies were not subjected to gene expression analysis.

Of the 497 fish analysed, 249 were found to be *sdY*-positive. Irrespective of age, *sdY* copy number was uniform amongst *sdY*-positive samples (~2500-5400 *sdY* copies/10 ng gDNA). However, amongst these samples, 10 had <5 *sdY* copies/10 ng gDNA and one had ~483 copies/10 ng gDNA.

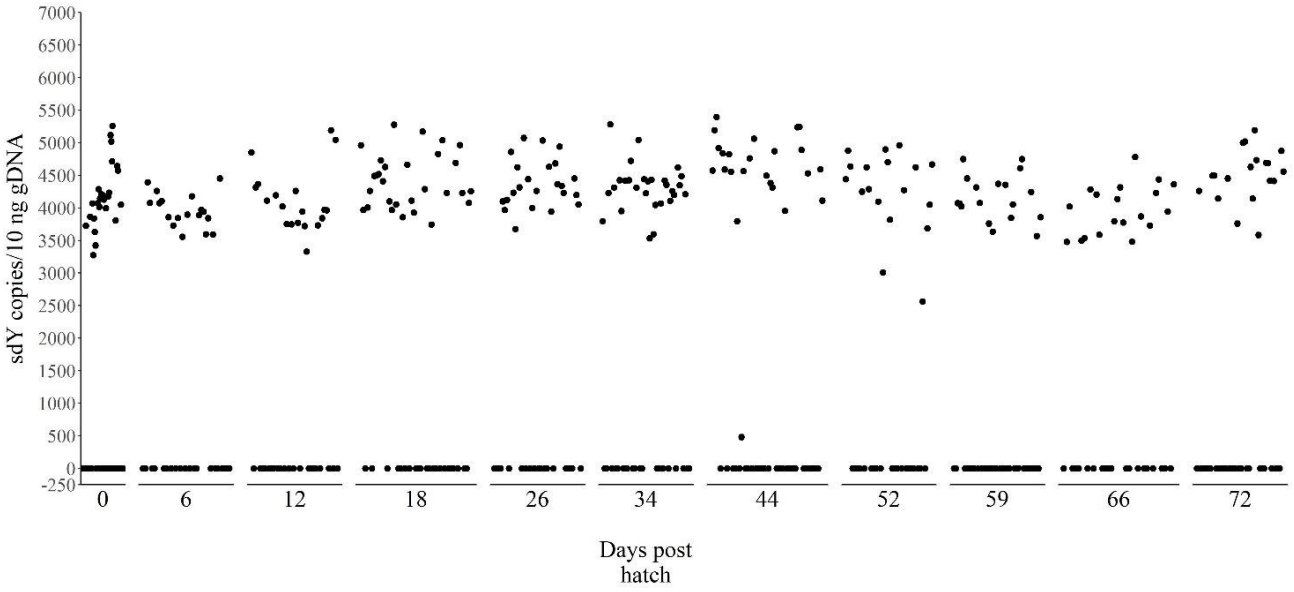

Figure 3. Quantification of sdY in gDNA from 497 Atlantic Salmon sampled at various ages during sex differentiation. The experimental population consisted of 8 families. ● indicates copy number for individual fish. 0 copies = female, >2,500 copies = male.

Growth

Fish increased in length at each sampling time during the experimental period. No sex-related differences in total length were observed at any sampling time (data not shown).

Gene expression

The expression of gonadal-related genes during sex differentiation demonstrated a male-dominant expression for amh, gsdf and sdY, and female-dominant expression for *cyp19a1a* (Fig. 4). Sex-related expression profiles were not observed for foxl2a or rspo1. The expression of *amh* in females was mostly undetectable throughout the study. In males, *amh* expression was practically undetectable before 34 dph, and increased thereafter to 72 dph. *amh* expression was significantly higher in males compared to females at 66 and 72 dph. Prior to 59dph, *gsdf* expression was hardly detectable in either sex. *gsdf* expression increased in both sexes at 66 dph and remained relatively constant thereafter. *gsdf* expression was significantly higher in males compared to females at 72 dph. *sdY* expression was not detected in females for the duration of the experiment. In males, *sdY* expression was barely detectable before 26 dph, but increased thereafter until 66 dph and then remained constant. *sdY* expression was significantly higher in males compared to females from 59 dph and thereafter. *cyp19a1a* expression was barely detectable in males throughout the duration of the experiment. In females, *cyp19a1a* expression was barely detectable until 59 dph, but it increased and remained relatively constant thereafter. *cyp19a1a* expression was significantly higher in females compared to males from 66 dph and thereafter. Irrespective of sex, *foxl2a* expression was highest at the time of hatch, and decreased until 52 dph where it remained constant

thereafter. *rspo1* expression was variable but remained relatively constant throughout the duration of the experiment.

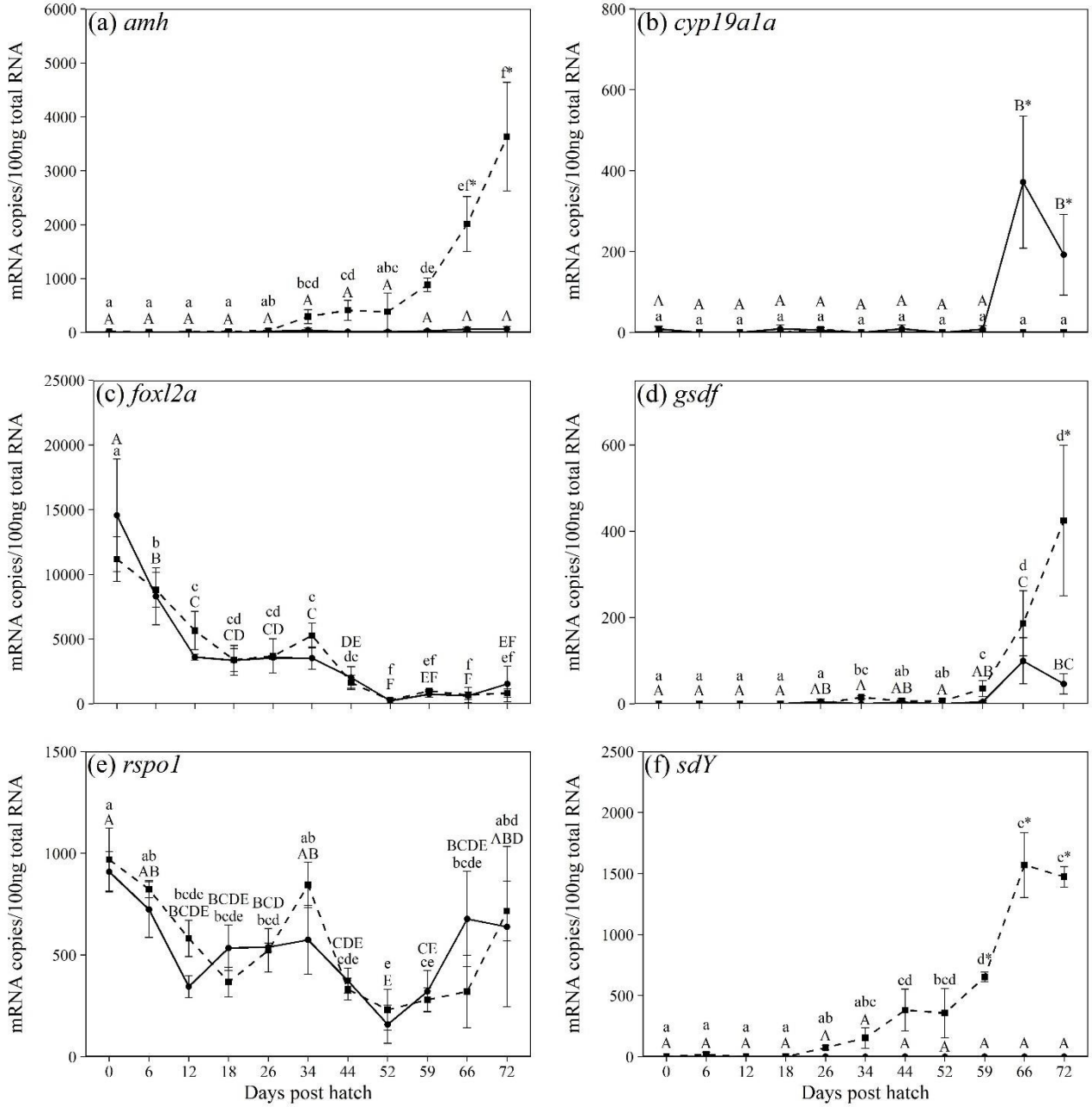

Figure 4. Mean (±S.E.M.) mRNA copies/100ng total RNA for amh, cyp19a1a, foxl2a, gsdf, rspo1 and sdY in trunks of female ●— and male —■— Atlantic Salmon at various ages during sex differentiation. Y-axis scales vary amongst these graphs. Significant differences ($p < 0.05$) within males are indicated by lowercase letters, and within females ($p < 0.05$) by different uppercase letters. * indicates significant differences between sexes within time ($p < 0.05$).

The expression of the neural gene *cyp19a1b* was detected in all sampling times, but there were no significant differences between males and females. Independent of sex, the expression of *cyp19a1b* was lowest from 0 to 18 dph and remained relatively constant after increasing at 26 dph (Fig. 5).

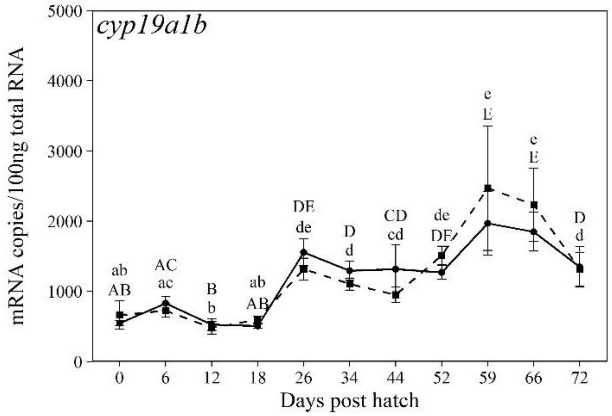

Figure 5. Mean (±S.E.M.) mRNA copies/100ng total RNA for cyp19a1b in heads of female ●– and male –■– Atlantic Salmon at various ages during sex differentiation. Y-axis scales vary amongst these graphs. Significant differences ($p < 0.05$) within males are indicated by lowercase letters, and within females ($p < 0.05$) by different uppercase letters. * indicates significant differences between phenotypes within time ($p < 0.05$).

The expression of the retinoic acid-signalling genes *aldh1a2*, *cyp26b1* and *tbx1a* was detected at all sampling times (Fig. 6). Independent of sex, the expression of these genes was highest at the time of hatch and decreased by 12 dph where it remained relatively constant thereafter. Exceptionally, *aldh1a2* expression increased to levels similar to the time of hatch at 72 dph. *cyp26a1* expression followed a similar trend, except levels were significantly higher in females than males at 66 dph.

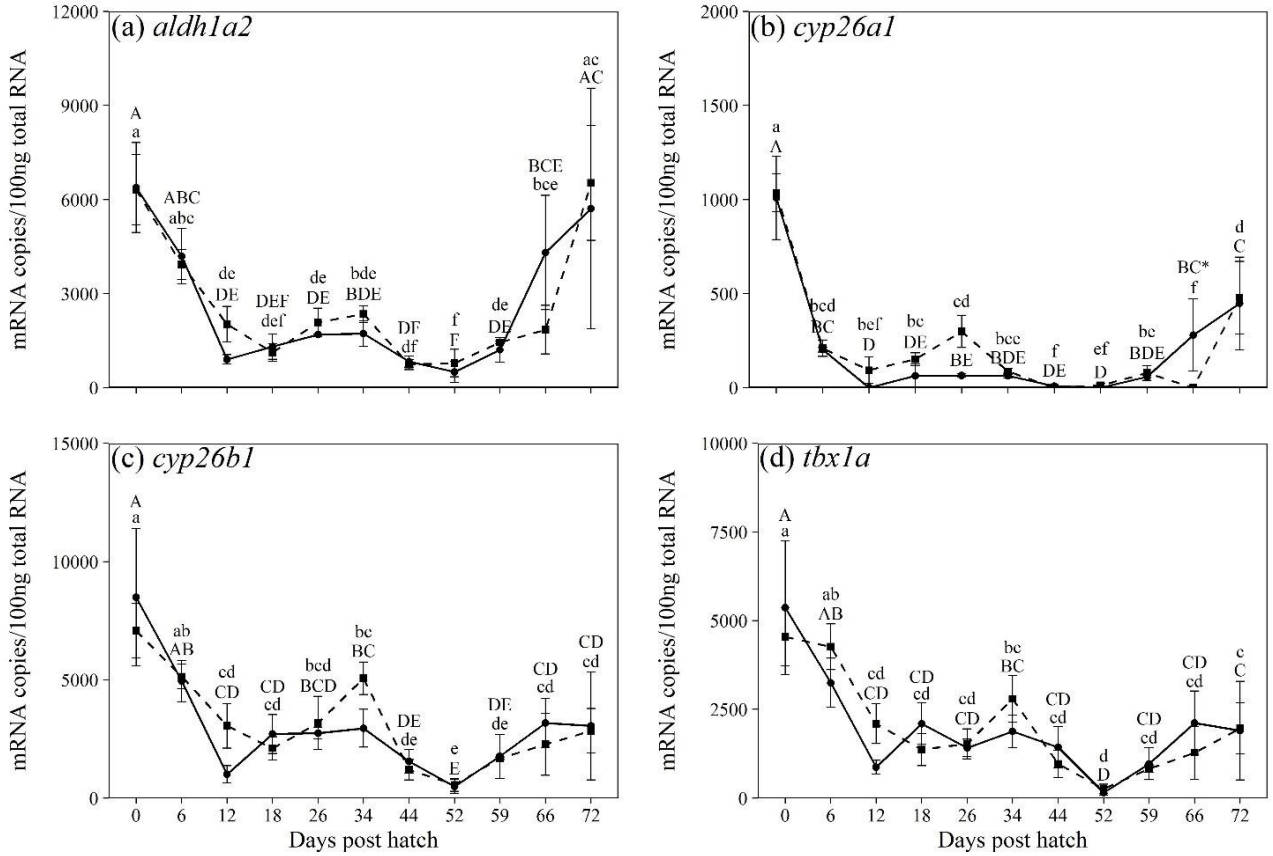

Figure 6. Mean (±S.E.) mRNA copies/100ng total RNA for aldh1a2, cyp26a1, cyp26b1 and tbx1a in trunks of female ●— and male —■— Atlantic Salmon at various ages during sex differentiation. Y-axis scales vary amongst these graphs. Significant differences ($p < 0.05$) within males are indicated by lowercase letters, and within females ($p < 0.05$) by different uppercase letters. * indicates significant differences between sexes within time ($p < 0.05$).

MDHT+DMSO experiment

Survival and growth

There were no significant differences in survival, total length and mass of genotypic female Atlantic Salmon at 81 dat (Table 1).

Table 1. Survival (%), total length (mm), fork length (mm) and mass (g) of genotypic female Atlantic Salmon following a 2-hour immersion in four experimental treatments at 19 days post hatch (dph). Survival was calculated from 3 days before hatch to 100 dph (81 days after treatment, dat). Total length and mass were recorded at 81 dat. Fork length and mass were recorded at 240-241 dat.

		81 days after treatment		**240-241 days after treatment**	
Treatment	**Survival (%)**	**Total length (mm)**	**Mass (g)**	**Fork length (mm)**	**Mass (g)**
Ethanol	75.86 ± 2.62[a]	45.35 ± 0.68 [a]	0.84 ± 0.04 [a]	130.91	29.88
DMSO	74.82 ± 2.68 [a]	44.92 ± 0.68 [a]	0.81 ± 0.04 [a]	130.13	28.75
MDHT	77.90 ± 2.72 [a]	44.82 ± 1.52 [a]	0.83 ± 0.08 [a]	135.18	32.48
MDHT+DMSO	80.84 ± 2.27 [a]	46.14 ± 0.66 [a]	0.87 ± 0.04 [a]	131.79	30.36

Values are presented as the mean ± S.E.M Significant differences ($p < 0.05$) between treatments are indicated by different lower-case letters.

Phenotypic sex ratio

Three distinct gonadal phenotypes were observed at 81 dat (Fig. 7), whereas four were observed at 240-241 dat (Fig. 8). At 81 dat, ovarian development was indicated by comparatively large gonads that were comprised of cysts of oocytes in meiotic prophase and the presence of an ovarian canal or ovarian cavity (Fig. 7a). Testicular development was indicated by comparatively small gonads that were comprised of singularly dispersed germ cells, mostly in the region distal to the mesentery, and clear formation of a vein, artery, and sperm duct in the proximal region (Fig. 7b). Gonads that did not clearly present sex-specific histological traits were considered to be undifferentiated. These gonads typically presented characteristics similar of testicular development except for absence of a well-defined sperm duct (Figure 7c). However, in many cases a network of lacunar spaces, which would be expected to later form the sperm duct (Nakamura, 1982; Foyle, 1993), were evident in the proximal region.

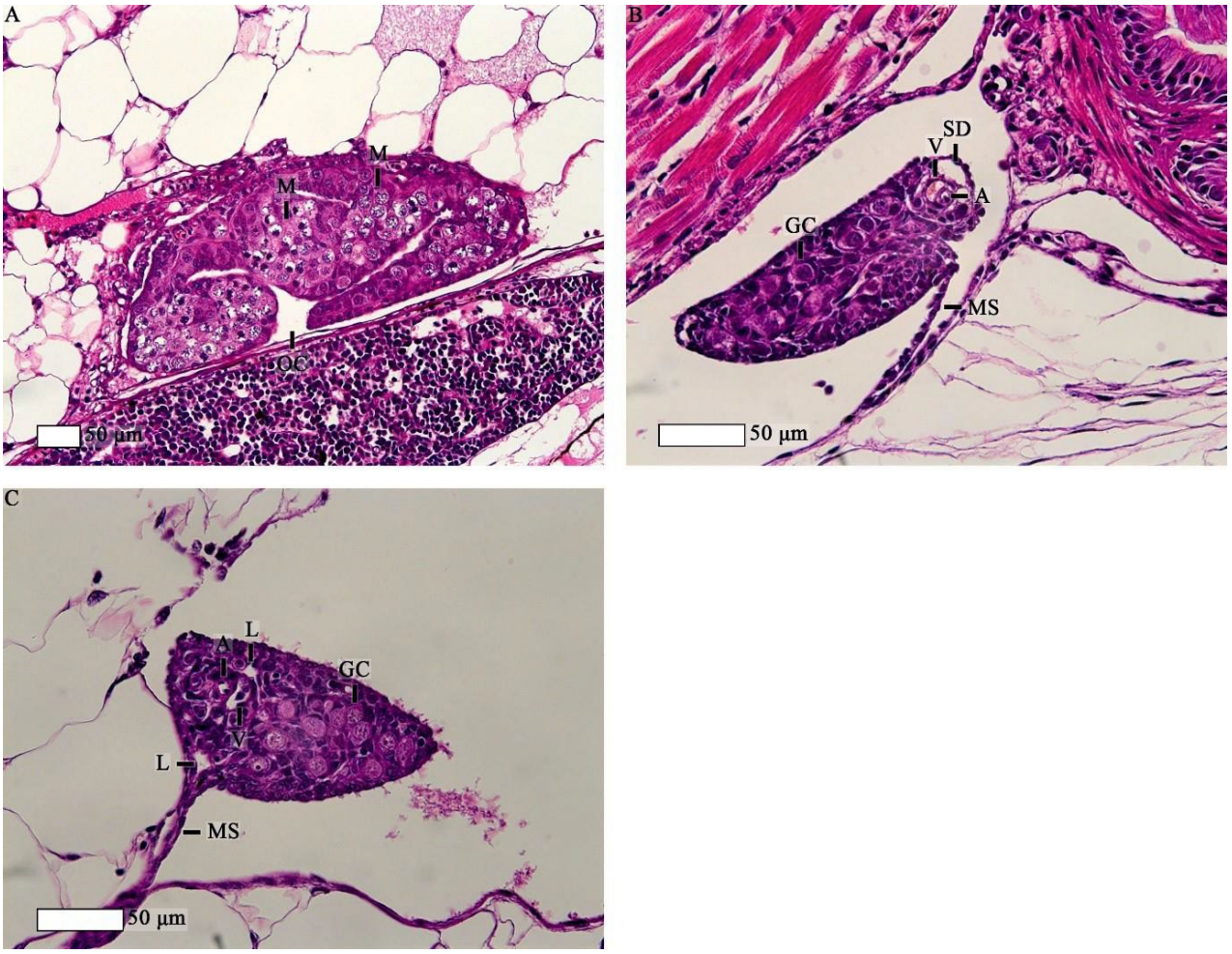

Figure 7. Gonadal phenotypes observed at 81 days after treatment in genotypic female Atlantic Salmon subjected to a 2-hour immersion in four experimental treatments at 19 days post hatch. A - Ovary comprised of cysts of oocytes in meiotic prophase (M), with ovarian canal (OC) evident. B - Testis comprised of singularly dispersed germ cells (GC), with a vein (V), artery (A) and sperm duct (SD) clearly formed in the region proximal to the mesentery (MS). C - Undifferentiated gonad comprised of singularly dispersed germ cells in the region distal to the mesentery, with a vein, artery, and lacunar spaces (L) evident in the proximal region.

At 240-241 dat, ovaries were predominantly comprised of oocytes in the early perinucleolus stage (Fig. 8a), whereas testes were comprised of cysts of type A and type B spermatogonia and Sertoli cells (Fig. 8b). Gonads that presented both ovarian and testicular structures were classified as intersex. Typically, these gonads were mostly comprised of cysts of spermatogonia but contained perinucleolar oocytes in the posterior region (Fig. 8c). Gonads that were almost entirely comprised of stromal tissue were classified as sterile (Fig. 8d).

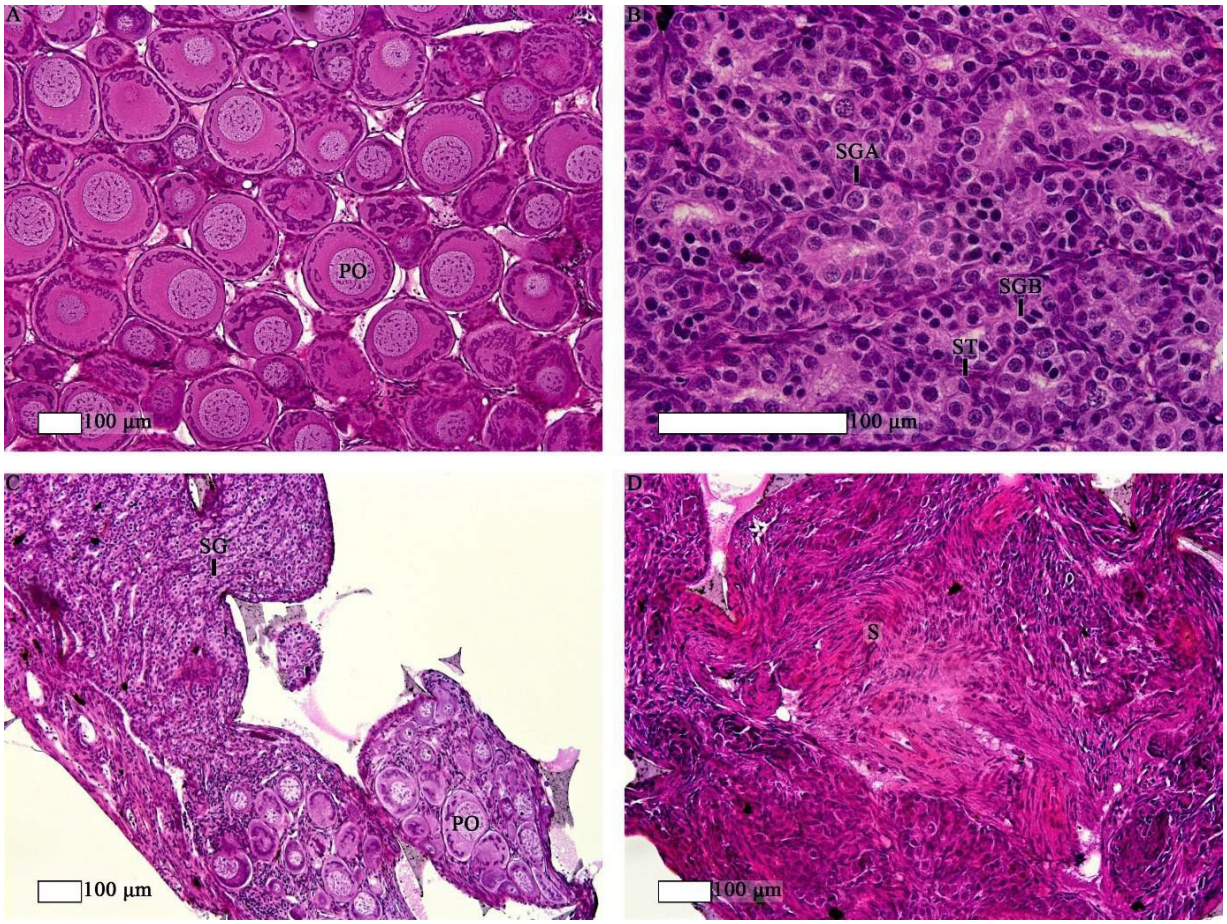

Figure 8. Gonadal phenotypes observed at 240-241 days after treatment in genotypic female Atlantic Salmon subjected to a 2-hour immersion in four experimental treatments at 19 days post hatch. A - Ovary comprised entirely of perinucleolar oocytes (PO). B - Testis comprised of cysts of type A (SGA) and type B (SGB) spermatogonia and Sertoli cells (ST). C - Intersex gonad mostly comprised of cysts of spermatogonia (SG), but perinucleolar oocytes present in posterior region. D - Sterile gonad that lacks germ cells and is entirely comprised of stromal tissue (S).

At 81 and 240-241 dat, the proportion of males (including undifferentiated fish at 81 dat) was similar between the control and DMSO groups, and similar between the MDHT and MDHT+DMSO groups (Table 2). The proportion of males (including undifferentiated fish at 81 dat) was higher in the MDHT and MDHT+DMSO groups compared to the control and DMSO groups at both sampling times (Table 2).

Table 2. Phenotypic sex proportions (%) in genotypic female Atlantic Salmon following a 2-hour immersion in four experimental treatments at 19 days post hatch. Phenotypic sex was determined at 81 and 240-241 days after treatment.

Treatment	81 days after treatment			240-241 days after treatment			
	Male (%)	Female (%)	Undifferentiated (%)	Male (%)	Female (%)	Intersex (%)	Sterile (%)
Control	0.00 ± 0.00	95.45 ± 4.55	4.55 ± 4.55	6.06	90.91	3.03	0.00
DMSO	0.00 ± 0.00	95.15 ± 5.01	4.85 ± 5.01	0.00	100.00	0.00	0.00
MDHT	33.54 ± 15.57	0.00 ± 0.00	66.46 ± 15.57	81.82	9.09	6.06	3.03
MDHT+ DMSO	21.51 ± 7.50	0.00 ± 0.00	78.49 ± 7.50	87.88	3.03	6.06	3.03

Values are presented as the mean ± S.E.M. At 81 days after treatment (dat), phenotype was assessed for 66, 62, 62 and 55 fish distributed amongst 3 replicates for control, DMSO, MDHT and MDHT+DMSO treatments, respectively. At 240-241 dat, phenotype was assessed for 33 fish for each treatment.

Gene expression

Gene expression results demonstrated that immersion in MDHT upregulated *amh* and *gsdf* expression, and downregulated *cyp19a1a* expression in genotypic female Atlantic Salmon. *foxl2a*, *rspo1* and *sdY* expression was unaffected by MDHT treatment (Fig. 9). The expression of *amh* and *gsdf* in fish at was low and relatively constant in the control group throughout most of the experimental period but increased at 56 days after treatment (dat). In the MDHT group, *amh* and *gsdf* expression was significantly higher than the control only at 56 dat.

The expression *cyp19a1a* in the control group was barely detectable until 3 dat and increased thereafter. In the MDHT group, *cyp19a1a* expression was barely detectable until 16 dat, and not detectable thereafter. Significant Differences between treatment groups for *cyp19a1a* expression were first evident at 33 dat. Irrespective of treatment group, *foxl2a* expression was highest on the day of treatment and remained relatively constant from 1 dat and thereafter. The expression of *rspo1* was not significantly different throughout the duration of the experiment. The expression of *sdY* was not detectable in either treatment group for the duration of the experiment.

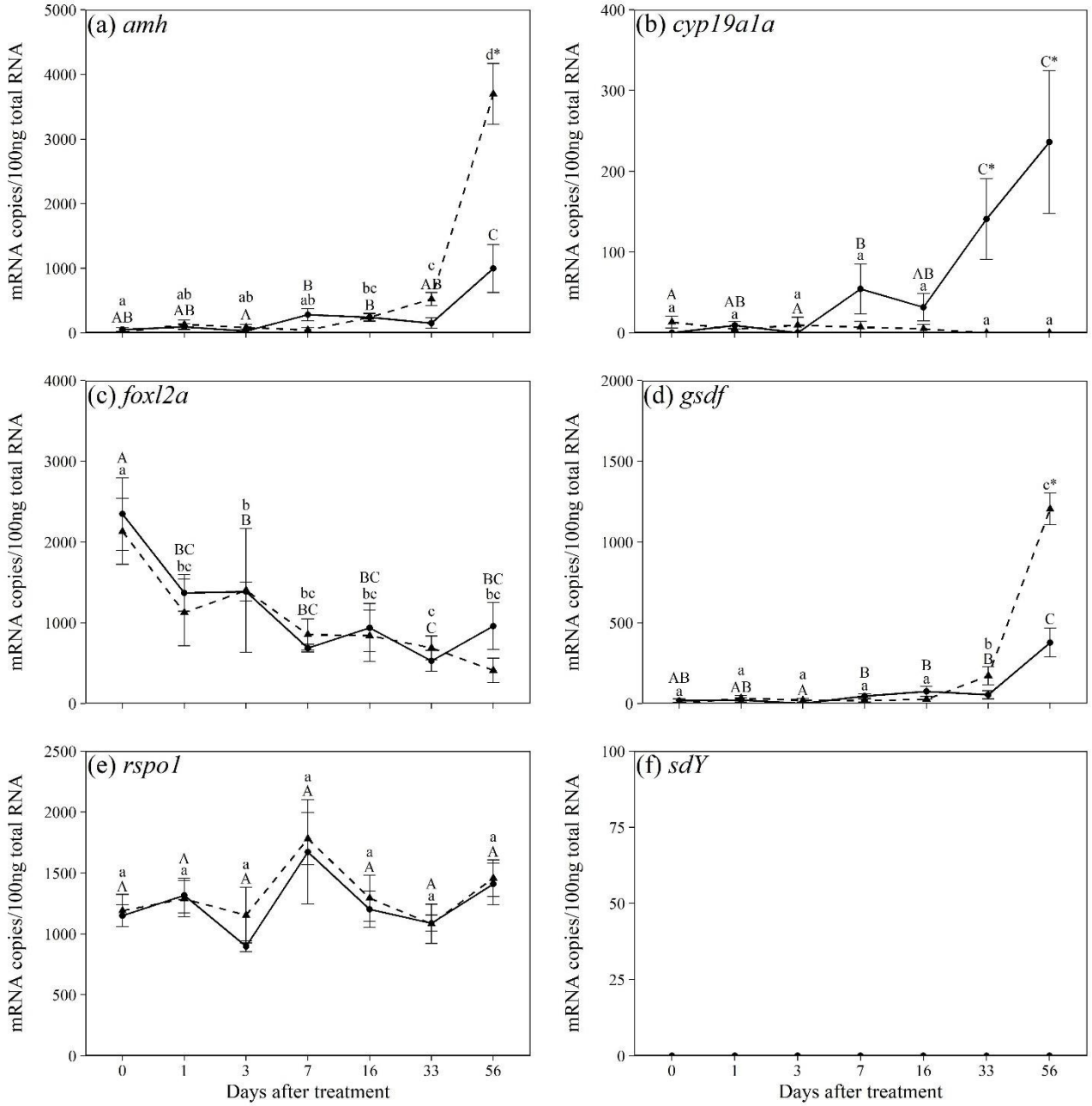

Figure 9. Mean (±S.E.) mRNA copies/100ng total RNA for amh, cyp19a1a, foxl2a, gsdf, rspo1 and sdY in genotypic female Atlantic Salmon trunks following a 2-hour immersion at 19 days post hatch in 17α-methyldihydrotestosterone (MDHT) -▲- (400 µg L^{-1} MDHT, 0.05 ppt ethanol) or control -●- (0 µg L^{-1} MDHT, 0.05 ppt ethanol). Y-axis scales vary amongst these graphs. Significant differences ($p < 0.05$) within the MDHT group are indicated by lowercase letters, and within the control group ($p < 0.05$) by different uppercase letters. * indicates significant differences between treatments within time ($p < 0.05$).

Aromatase Inhibitor Letrozole

Phenotypic sex ratio

At 257 dph, the sex ratio of the letrozole treatment groups was not different to the control. 100% female populations were observed in all groups (data not shown).

Discussion

Sex differentiation experiment

Detailed histological descriptions of sex differentiation have been provided for a number of salmonid species, including coho salmon (*Oncorhynchus kisutch*) (Foyle, 1993), whitespotted charr (*Salvelinus leucomaenis*) (Nakamura, 1982), brook trout (*Salvelinus fontinalis*) (Fatima et al., 2012) and rainbow trout (*Oncorhynchus mykiss*) (Van den Hurk and Slof, 1981; Lebrun et al., 1982). In these species, one of the key histological events indicating sex differentiation is meiotic entry in ovarian but not testicular germ cells. Detailed histological descriptions are currently lacking in Atlantic Salmon, but in this species meiosis in ovarian germ cells seems to initiate approximately 60 dph (1050-degree days post fertilisation, ddpf) (Laird et al., 1978). In coho salmon and rainbow trout, the onset of meiosis during ovarian differentiation has been reported at 800 ddpf, and 610 ddpf, respectively (Nakamura, 1982; Foyle, 1993; Feist et al., 1996; Baron et al., 2005). In the present study, we investigated histological gonad development from 18 dph (614 ddpf) to 79 dph (1177 ddpf) in a mixed sex Atlantic Salmon population. Throughout our study, no germ cells were observed to have entered meiosis. These findings suggest histological sex differentiation occurs later in Atlantic Salmon than previously reported (Laird et al., 1978), and considerably later than in *Oncorhynchus* spp.

In addition to earlier onset of meiosis, ovarian differentiation in salmonids is also characterised by increased germ cell number and gonad size, clustering of germ cells and formation of an ovarian canal (Van den Hurk and Slof, 1981; Lebrun et al., 1982; Nakamura, 1982). At 79 dph, germ cells were arranged in cysts in all genotypic females analysed. Gonad cross-section area and germ cell number tended to be higher in females compared to males at this time (although not statistically significant), and an ovarian canal was evident in 1 of 5 females. These findings demonstrate that histological sex differentiation was evident at this time (79 dph), despite meiotic germ cells not being present. Interestingly, the ovarian canal is not formed until after meiotic germ cells become evident in coho salmon (Foyle, 1993) and whitespotted charr (Nakamura, 1982). This is in contrast with what we observed in Atlantic Salmon. Germ cells were also clearly arranged in cysts in 6 of 12 mixed-sex fish at 66 dph and 3 of 12 mixed-sex fish at 52 dph, suggesting histological sex differentiation is becoming evident at these ages as well. However, no differences in gonad cross-section area or germ cell number were evident between phenotypes at these stages. Unfortunately, genotypic sex could not be determined in these Bouin's-fixed samples due to DNA degradation caused by fixation. As such, we could not determine the proportion of genotypic females for which female phenotype was evident, nor could we verify that female phenotype was assigned to only genotypic females. Despite this, our findings suggest histological sex differentiation in Atlantic Salmon is first evidenced by sex-related germ cell arrangement and may become evident from as early as 52 dph.

We investigated temporal and sex-related expression patterns for several sex differentiation-related genes during the experimental period. In addition to numerous other teleost species, these genes have been previously studied in rainbow trout, and except for *rspo1*, have generally been demonstrated to exhibit sex-related expression shortly after hatching in this species (Baron et al., 2007; Vizziano et al., 2007; Nicol and Guiguen, 2011; Yano et al., 2012). In other teleosts, such as Nile tilapia (*Oreochromis niloticus*) (Wu et al., 2016) and medaka (*Oryzias latipes*) (Chakraborty et al., 2016), *rspo1* is upregulated in females only before histological sex differentiation becomes evident.

In contrast to rainbow trout, none of the genes investigated showed sex-related expression close to the time of hatching. Sex-related expression was first observed at 26 dph, with *sdY* becoming upregulated in males. At 34 dph, *amh* was also upregulated in males. *sdY* has been evidenced as the master sex determining gene in rainbow trout (Yano et al., 2012) and is strongly associated with male phenotype in Atlantic Salmon (Brown et al., 2020). Our findings indicate that *sdY* could be one of the first genes upregulated in male Atlantic Salmon during sex differentiation and are in support of a hypothesised role as a master sex determinant in this species. Considering their expression profiles were similar in males during the experimental period, *sdY* may promote testicular differentiation in part via inducing *amh*. *gsdf* expression was also highest in males, but only at 72 dph. This appears to be later than the initiation of histological sex differentiation, suggesting *gsdf* is not involved in triggering testicular fate. *gsdf* does not

have a critical role during testicular differentiation in zebrafish (*Danio rerio*) either (Yan et al., 2017), but *gsdf*-knockout in Nile tilapia (Jiang et al., 2016) and medaka (Zhang et al., 2016; Guan et al., 2017) results in complete population male-to-female sex reversal. In medaka, *gsdf* transcription during sex differentiation is directly induced by *dmy*, the master sex determining gene in this species (Zhang et al., 2016). In contrast, *dmrt1* regulates *gsdf* transcription in Nile tilapia and spotted scat (*Scatophagus argus*). As *dmrt1* expression is not sex-related during sex differentiation in Atlantic Salmon, it remains unclear which factors regulate *gsdf* expression in this species (Lubieniecki et al., 2015).

cyp19a1a encodes for production of aromatase, an enzyme that catalyses the synthesis of estrone and estradiol from androstenedione and testosterone, respectively (Meinhardt and Mullis, 2002). *foxl2* functions in the transcriptional regulation of *cyp19a1a* during ovarian differentiation (Wang et al., 2007; Yamaguchi et al., 2007). Except for medaka, who possess estrogen-independent ovarian differentiation (Suzuki et al., 2004; Nakamoto et al., 2018), estrogen is generally regarded to have a critical role during ovarian differentiation. In Nile tilapia, *cyp19a1a* and *foxl2* are required for ovary-specific increases in germ cell number (Zhang et al., 2017). *cyp19a1a* and *foxl2a* have been shown to present highly correlated expression patterns in rainbow trout (Baron et al., 2004), turbot (*Scophthalmus maximus*) (Ribas et al., 2016), Japanese flounder (*Paralichthys olivaceus*) (Yamaguchi et al., 2007), medaka (Nakamoto et al., 2006) and Nile tilapia (Ijiri et al., 2008). In rainbow trout, these genes are upregulated in females shortly after hatching, like *sdY* and *amh* in males. It is surprising therefore that in our study, *cyp19a1a* was not upregulated in female Atlantic Salmon until 66 dph and *foxl2a* expression was not sex related. As *cyp19a1a* was upregulated in females prior to sex-related differences in germ cell number, estrogens could function in ovary-specific germ cell proliferation in Atlantic Salmon as demonstrated in Nile tilapia (Zhang et al., 2017). However, formation of germ cell-cysts appeared to be evident before this time, suggesting other factors may be involved in driving initial stages of histological sex differentiation in the ovary. The low levels of *cyp19a1a* expression observed raise questions regarding the functional significance of *cyp19a1a* during sex differentiation. Our results may also suggest that *foxl2a* is not involved in ovarian differentiation in Atlantic Salmon, although it seems likely that as gene expression was analysed in trunks rather than isolated gonads, expression from non-gonadal tissues could be masking sex-related differences within the gonad. Immunohistochemical analysis of Foxl2 and aromatase will be valuable in answering these questions. Lack of sex-related *rspo1* expression observed in this study may suggest that the function of *rspo1* during sex differentiation in Nile tilapia and medaka is not shared in salmonid species.

A recurrent interest in the field of teleost sex differentiation has been the role, if any, of extra-gonadal tissues. The role of the brain via *cyp19a1b* (a *cyp19* isoform predominantly localised in the brain), and subsequently neuroestrogens on sex differentiation has received some attention [see (Blázquez and Somoza, 2010) for previous review]. *cyp19a1b* expression patterns during sex differentiation are not strictly conserved amongst teleost fish. Furthermore, *cyp19a1b* loss-of-function studies in zebrafish (Yin et al., 2017) and Nile tilapia (Zhang et al., 2019) have not revealed any effects on phenotypic sex. Such findings are in agreement with the absence of sex-related differences in *cyp19a1b* expression or neural aromatase activity during sex differentiation in these species (Kwon et al., 2001; Sudhakumari et al., 2005; Kallivretaki et al., 2007). Our findings indicate *cyp19a1b* expression is not sex-related during sex differentiation in Atlantic Salmon. These observations further suggest *cyp19a1b* lacks a role during sex differentiation in teleost fish. However, they contrast those made in rainbow trout, where male-dominant *cyp19a1b* expression and neural aromatase activity during sex differentiation has been reported (Vizziano-Cantonnet et al., 2011).

Aldehyde dehydrogenase (Aldh1a) and cytochrome P450 family 26 (Cyp26) enzymes regulate the availability of retinoic acid (RA) via its synthesis and degradation, respectively (Pennimpede et al., 2010). RA, the active metabolite derived from vitamin A, has been implicated in meiotic initiation in vertebrates [see Agrimson and Hogarth (2016) for review]. Such a role has specifically been evidenced in teleosts, where exogenous RA and ketoconazole (Cyp26 inhibitor) treatments advance meiotic entry, and 4-diethylaminobenzaldehyde (Aldh1a inhibitor) treatments delay meiotic entry in ovarian germ cells during sex differentiation (Feng et al., 2015; Li et al., 2016; Peng et al., 2020). In the present study, expression of *aldh1a2*, *cyp26a1* and *cyp26b1* were not sex-related during the experimental period. In contrast, *aldh1a2* expression is upregulated and *cyp26a1* expression is downregulated in female Nile tilapia during sex

differentiation at the onset of meiosis (Feng et al., 2015). Similar findings have also been demonstrated for *aldh1a2* and *cyp26* genes in southern catfish (*Silurus meridionalis*) (Li et al., 2016) and Japanese flounder (Yamaguchi and Kitano, 2012). These genes need to be investigated in Atlantic Salmon at developmental stages coinciding with onset of meiosis in the differentiating ovary before a role in sex differentiation can be disregarded in this species.

tbx1a is known to be regulated by RA (Yano 2011). Our observations of similar temporal expression profiles for *tbx1a, aldh1a2*, *cyp26a1* and *cyp26b1* support that these genes are involved in similar pathways. In rainbow trout, *tbx1a* is expressed higher in males compared to females shortly after hatching. This pattern is maintained throughout the sex differentiation period (Yano et al., 2011). In contrast, we did not observe sex-related expression for *tbx1a* during sex differentiation in Atlantic Salmon.

MDHT + DMSO experiment

Our study produced an 82% male Atlantic Salmon population from an all-female population following a single 2-hour immersion in MDHT at 19 dph. This is the highest rate of masculinisation reported in Atlantic Salmon following a single immersion treatment. A previous study involving all-female Atlantic Salmon reported production of 11%, 58% and 61% male populations following a single 2-hour immersion in MDHT (400 µg L^{-1}, dissolved in ethanol) at 0 dph, 7 dph and 14 dph, respectively (Lee et al., 2004). Taken together, these findings indicate that the effectiveness of an MDHT immersion treatment in Atlantic Salmon increases with age that the treatment is administered.

In our study, MDHT treatment completely suppressed *cyp19a1a* expression in female Atlantic Salmon, whereas *foxl2a* and *rspo1* expression was unaffected. Previous studies have demonstrated *cyp19a1a* expression is also reduced following exogenous androgen treatment in female rainbow trout (diet, 11β-hydroxyandrostenedione (11βOHΔ4) (Baron et al., 2008; Vizziano et al., 2008)), but not in zebrafish (immersion, MT (Lee et al., 2017)). In contrast to our study, expression of *foxl2* [a transcriptional regulator of *cyp19a1a* in teleost fish (Wang et al., 2007; Yamaguchi et al., 2007; Fan et al., 2019)] was reduced in a similar manner to *cyp19a1a* in rainbow trout (diet, 11βOHΔ4 (Baron et al., 2008; Vizziano et al., 2008)). *foxl2* expression was also reduced in Hong Kong catfish (*Clarias fuscus*) (diet, MT (Deng et al., 2015)), but not zebrafish (immersion, MT (Lee et al., 2017)) following treatment with an exogenous androgen. Absence of an effect of MDHT on *foxl2a* and *rspo1* expression in the present study is in agreement with the lack of sex-related differences reported for these genes during natural sex differentiation in Atlantic Salmon (Lubieniecki et al., 2015; Sex differentiation experiment). As the studies of Lubieniecki et al., (2015) and the present study examined gene expression in Atlantic Salmon trunks or whole embryo's rather than isolated gonad tissue, it is possible that expression from non-gonadal tissues could be masking sex and treatment-related differences within the gonad. Analysis of isolated gonads, in-situ hybridisation and/or immunohistochemical approaches are now needed to determine whether sex and treatment-related differences in *foxl2a*/Foxl2a and *rspo1*/Rspo1 expression exist in the gonads. Only then can a role of *foxl2a* and *rspo1* (or lack of) during sex differentiation and sex reversal in Atlantic Salmon be inferred.

In the present study, *amh* and *gsdf* expression was upregulated in female Atlantic Salmon following MDHT treatment. The age at which these genes became upregulated in females following MDHT treatment, and their expression levels, are similar to that of genotypic males during sex differentiation (Sex differentiation experiment). Increased *amh* expression was also demonstrated in zebrafish following treatment with an exogenous androgen (immersion, MT (Lee et al., 2017)), whereas increased *gsdf* expression has been demonstrated in zebrafish (immersion, MT (Lee et al., 2017)) and northern medaka (*Oryzias sakaizumii*) (immersion, MT (Horie et al., 2016)). In female rainbow trout, *gsdf* expression is unaffected following treatment with an exogenous androgen (diet, 11βOHΔ4 (Baron et al., 2007)) and *amh* is downregulated (diet, 11βOHΔ4 (Baron et al., 2008; Vizziano et al., 2008)). These exceptional findings in rainbow trout could be linked to the nature of dietary treatments (age at exposure, frequency, and duration of exposure). Baron et al., (2007), Vizziano et al., (2008) and Baron et al., (2008) found that the transcriptional pathways underpinning female-to-male sex reversal in rainbow trout following dietary treatment with 11βOHΔ4 were vastly different from those involved in natural testicular differentiation. In contrast, although limited to a

small number of genes, our findings suggest testicular promoting pathways are similar between genotypic female Atlantic Salmon subjected to an immersion in MDHT and genotypic males. An effect of administration route on gene expression could be expected considering gonad malformations are imparted in a high proportion of Atlantic Salmon (Johnstone and Youngson, 1984; de Castro and Patil, 2019) and rainbow trout (Johnstone et al., 1979; Cousin-Gerber et al., 1989) neo-males produced by dietary exogenous androgen treatments, but not immersion treatment regimens (Lee et al., 2004). An effect of the type and dose of exogenous androgen used cannot be disregarded either. More studies are needed to determine how these variables (age at exposure, frequency and duration of exposure, hormone type and dose) affect the mechanisms underpinning sex reversal in teleost fish. Lack of *sdY* expression in either experimental group from the present study is concordant with the fact that only genotypic female Atlantic Salmon were used in the experiment.

Effects of MDHT treatment in our study were first evident at 33 dat by down regulation of *cyp19a1a* expression, and then at 56 dat by upregulation of *amh* and *gsdf*. There were no effects evident at 16 dat. The physiological significance of reduced *cyp19a1a* expression at 33 dat and 56 dat following MDHT treatment is unknown considering only low expression levels were detected in the control group. In this respect, further study is needed to determine whether *cyp19a1a* expression levels observed in female Atlantic Salmon at these ages translates to synthesis of aromatase and subsequently endogenous estrogen production. Nonetheless, our findings indicate a significant delay in the effects of exogenous androgen treatment compared to previous studies. For example, effects on gene expression were evident from 3 dat in female Nile tilapia (diet, MT (Kobayashi et al., 2008)), 7 dat in female rainbow trout (diet, 11βOHΔ4 (Baron et al., 2008; Vizziano et al., 2008)) and 10 dat in Hong Kong catfish (diet, MT (Deng et al., 2015)). The apparent delay in treatment effects observed in the present study could be due to the fact that MDHT treatment was applied well before sex-related gene expression is initiated in Atlantic Salmon (Lubieniecki et al., 2015; Sex differentiation experiment). This idea lends itself to the hypothesis that the embryonic yolk sac acts as a reservoir for lipophilic exogenous steroids up taken during an immersion treatment and permits continued exposure well after the immersion treatment has ceased (Piferrer and Donaldson, 1994). In the case of our study, the yolk sac may facilitate continued exposure to MDHT until endogenous sex differentiation pathways become activated, and therefore labile to its effects. As the first effects of MDHT are evident from 52 dph in female Atlantic Salmon, we further hypothesise that performing immersion treatments closer to this age will result in higher rates of sex reversal than currently obtained. In addition to immersion timing, further refinement of immersion duration and dose may be needed for a single MDHT immersion treatment to be sufficient for complete population masculinisation in Atlantic Salmon. An alternative hypothesis is that other genes not investigated in the present study are activated/repressed following the exogenous steroid treatment, and subsequently cause delayed changes in *amh*, *cyp19a1a* and *gsdf* expression. These hypotheses are not necessarily mutually exclusive. Use of transcriptomic approaches in the future will provide greater insights into the time course and extent of changes in gene expression during exogenous androgen-induced female-to-male sex reversal.

Our study tested whether inclusion of DMSO as a solvent in addition to ethanol increased the effectiveness of a single 2-hour immersion treatment in MDHT. Immersion in MDHT (400 µg/L) and DMSO (300 ppm) produced an 88% male population Atlantic Salmon population from an all-female population. This rate of masculinisation is the highest reported in Atlantic Salmon following a single 2-hour immersion treatment but was not significantly different from that achieved following immersion in MDHT (400 µg/L) alone (82%). In bluegill (*Lepomis macrochirus*), inclusion of DMSO in an immersion treatment with trenbolone acetate increased the proportion of males by 6% compared to the same treatment without DMSO (Arslan and Phelps, 2003). This increase in masculinisation is similar to that observed in the present study. DMSO would be expected to increase the effectiveness of an immersion treatment by facilitating uptake of the exogenous steroid hormone. Therefore, further insights could be gained by studying the effects of DMSO on steroid hormone uptake (during the immersion) and retention (following the immersion). Inclusion of DMSO in the immersion treatments in the present study did not affect survival or growth, supporting its potential for use in sex reversal treatments.

In our study, female phenotype was not evident in any of the fish treated with MDHT at 81 dat (Table 2). At 240-241 dat, 9% and 3% of fish sampled from the MDHT and MDHT+DMSO treated groups, respectively,

were phenotypically female. Additionally, 6% were intersex and 3% sterile in each of these groups. These findings suggest that for a small proportion of fish in these groups, sex reversal evident at 81 dat was transient and gonadal phenotype partially or completely reverted back to the genotypic sex by 240-241 dat. Transient sex reversal has been previously reported in salmonids following treatment with steroid hormones and endocrine disrupting chemicals. The rate of transient sex reversal in the present study was similar to that reported by Olito and Brock (1991). In this study, dietary MT treatment induced complete population masculinisation (identified mostly by undifferentiated gonads) in rainbow trout when phenotypic sex ratio was determined one month after treatment (Olito and Brock, 1991). Re-examination of phenotypic sex seven months after treatment further confirmed the absence of females, but 10% of fish sampled were intersex or lacked gonadal tissue (Olito and Brock 1991). Alternatively, higher rates of transient sex reversal have been reported by Van den Hurk and Van Oordt (1985) and Afonso et al., (2002). Van den Hurk and Van Oordt (1985) observed high levels of masculinisation (94-100%) at 150 dpf in a mixed-sex population of rainbow trout following dietary or immersion treatment with 11βOHΔ4. Re-examination of the phenotypic sex at 300 dpf revealed no significant difference between the proportion of males in the immersion treatment group (52%) and the control (46%), whereas the proportion of males in the dietary treatment groups was reduced (76-78%) (Van den Hurk & Van Oordt, 1985). A study by Afonso et al., (2002) found that exposure to bleach kraft mill effluent or primary sewage effluent in chinook salmon (*Oncorhynchus tshawytscha*) had disturbed testicular development (sex reversed, intersex, or reduced/lack of germ cells) in 95-100% of genotypic males observed at 103 dph. When these groups were re-examined at 179 dph, only 0-14% of genotypic males showed disturbed testicular development (Afonso et al., 2002). Taken together, these studies highlight the importance of conducting long-term experiments when studying sex reversal in salmonids (Afonso et al., 2002).

A small proportion of male and intersex fish were observed at 240-241 dat in the control group in our study. Ovarian development also appeared to be retarded in some individuals from the control and solvent control groups at 81 dat. We confirmed that fish sampled at 240-241 dat were genotypic females (*sdY*-negative) using the real-time qPCR method outlined by Brown et al., (2020). As immersions in ethanol did not affect phenotypic sex in Nile tilapia (Wassermann and Afonso, 2003), rainbow trout (Feist et al., 1995) or brook trout (Fatima et al., 2016), our findings suggest a small proportion of genotypic female Atlantic Salmon develop as phenotypic males or intersex naturally. Indeed, small proportions (1-8%) of phenotypic males have been previously reported in all-female Tasmanian Atlantic Salmon populations (Lee et al., 2003; Lee et al., 2004), whereas intersex fish have been identified in populations of brown trout (*Salmo trutta*) (Bjerregaard et al., 2008) and brook trout (Galbreath and Stocks, 1999). More recently, studies in Tasmanian (Eisbrenner et al., 2014) and Norwegian (Perry et al., 2019) Atlantic Salmon populations have observed small proportions of genotypic females with male phenotype. Taken together, these findings suggest an influence of alternative factors in addition to *sdY* on Atlantic Salmon sex determination. Interestingly, no male or intersex fish were observed in the solvent control group at 240-241 dat. This could suggest a feminising effect of DMSO when administered alone.

Aromatase Inhibitor Letrozole

Sex differentiation in *Oncorhynchus* spp. can be affected by treatment with exogenous estrogen around the time of hatching (Piferrer and Donaldson, 1989, 1992; Razmi et al., 2011). It has also been demonstrated in chinook salmon that sex differentiation can be affected by a single 2-hour immersion treatment with a non-steroidal aromatase inhibitor (fadrozole, 10 mg L^{-1}) at 3 dph. Together, these findings suggest *Oncorhynchus* spp. are sensitive to estrogen manipulation around the time of hatching. In agreement with this, female-dominant expression of *cyp19a1a*, which encodes the gonadal aromatase enzyme and indicates synthesis of endogenous estrogen, has been demonstrated at this time in rainbow trout (Bertho et al., 2018; Vizziano et al., 2007). In the present study two 6-hour immersion treatments in letrozole (10 mg/L), applied 8 days apart and initiated either 7 days before hatch, 1 dph or 9 dph did not induce female-to-male sex reversal in Atlantic Salmon. Similarly, female-to-male sex reversal was not observed in all-female Atlantic Salmon subjected to two 2-hour immersions in fadrozole (10 mg L^{-1} and 50 mg L^{-1}), either at 7 and 14 dph or 14 and 21 dph (Lee et al., 2003). These findings suggest unlike *Oncorhynchus* spp., Atlantic Salmon may not be sensitive to estrogen manipulation around the time of hatching. This is further

evidenced by the findings that *cyp19a1a* gene expression was not significantly different between males and females prior to the time of first feeding in Atlantic Salmon (Lubieniecki et al., 2015; Sex differentiation experiment), indicating considerable differences in the timing of activation of endogenous estrogen synthesis pathways in comparison to rainbow trout. Taken together, immersion treatments with non-steroidal aromatase inhibitors may be effective in Atlantic Salmon when conducted at a time synchronous with activation of endogenous estrogen synthesis in females.

Conclusion

In summary, this project has characterised aspects of sex differentiation at the morphological and gene expression level in Atlantic Salmon. Sex-related differences in gonad morphology were clearly established by 79 dph. At this stage, ovarian differentiation was evident by the formation of germ cell cysts throughout the entire length of the gonad cross-section, and in one individual also by the ovarian canal. Testicular differentiation was indicated by singularly dispersed germ cells located within the distal region of the gonad cross-section. Gonad cross-section area and germ cell number tended to be higher in the ovary compared to the testis at 79 dph, whereas germ cell diameter tended to be lower. Sex-related differences in gene expression were first evident at 59 dph by male-dominant *sdY* expression. Thereafter, testicular differentiation was characterised by male-dominant expression of *amh* and *gsdf*, and ovarian differentiation by female-dominant expression of *cyp19a1a*. Sex related differences in the expression of *cyp19a1b*, *foxl2a*, *rspo1* and RA-signalling genes were not observed during the experiment.

This project has also provided new insights into the mechanisms, and their time course, during exogenous androgen-induced female-to-male sex reversal in Atlantic Salmon. The findings suggest that the effects of an immersion treatment in MDHT at 19 dph are delayed but induce gene expression profiles similar to those exhibited by genotypic males during testicular differentiation. This is characterised by a maintained suppression of *cyp19a1a* expression and upregulation *amh* and *gsdf* expression. The present study also demonstrated production of an 88% male Atlantic Salmon population from an all-female population following a single 2-hour immersion in MDHT (400 µg L^{-1}) and DMSO (300 mg L^{-1}) at 19 dph, which is the highest rate of sex reversal achieved in Atlantic Salmon following a single immersion treatment. It remains unclear whether DMSO increased the effectiveness of the single immersion treatment.

Implications and recommendations

Our study has provided new insights into the timing of histological and transcriptional sex differentiation in Atlantic Salmon. Our findings have demonstrated that sex differentiation in Atlantic Salmon occurs later than previously reported. Meiotic initiation in the ovary, which is a key indicator of histological sex differentiation, was not observed in the present study. It therefore occurs later than 79 dph in Atlantic Salmon. We showed that histological sex differentiation in this species is evident well before onset of meiosis however, first by sex-specific germ cell arrangement, followed by sex-related changes in germ cell diameter, germ cell number, gonad cross section size and formation of the ovarian canal. Interestingly, histological sex differentiation appears to become evident before sex-specific expression of several important sex differentiation-related genes. Many of the sex differentiation-related genes we, and previous studies (Lubieniecki et al., 2015) have investigated in Atlantic Salmon appear to lack sex-related expression during sex differentiation. As a result, the genes regulating sex differentiation in Atlantic Salmon are mostly unknown and further study is needed. Transcriptomic approaches may be valuable here. It is also possible that sex-related differences in the gonad are being masked by non-gonadal tissues when trunk or whole embryos are used. Due to the difficulty of dissecting gonads from Atlantic Salmon during the sex differentiation period, in situ-hybridisation and immunohistochemistry approaches will be useful for determining this. Another finding of interest is the apparent differences between rainbow trout and Atlantic Salmon in the onset of female-dominant *cyp19a1a* expression. *cyp19a1a* is upregulated in female

rainbow trout around the time of hatching, but in Atlantic Salmon not until after time of first feeding. This may explain why aromatase inhibitor treatments similar to those used in *Oncorhynchus* spp. (administered around time of hatch) are not effective in Atlantic Salmon [see Lee et al., (2003) and 'Letrozole experiment'], as in Atlantic Salmon endogenous aromatase is not being synthesised at this stage, and therefore cannot be inhibited. This realisation will be particularly important for the Tasmanian Atlantic Salmon aquaculture industry in their pursuit for developing a non-steroidal sex reversal method. Our results suggest treatments should be administered around the time of first feeding.

Establishing a DNA-based method that reliably predicts phenotypic sex in Atlantic Salmon remains of high interest in the aquaculture setting. Such a method will enable use of more desirable alternatives to the dietary sex reversal methods currently used (Lee et al., 2004). *sdY* presents as a promising DNA-based sex marker in this species, however low rates of erroneous alignment with *sdY* and phenotypic sex have been consistently reported in the Tasmanian strain (Eisbrenner et al., 2014; Kijas et al., 2018; Brown et al., 2020), thereby preventing such use. In our recent study we found that discordant phenotypic females could be distinguished from concordant phenotypic males by a reduced *sdY* copy number, possibly indicating a mosaic presence of the gene in these individuals. In contrast, another recent study in Norwegian Atlantic Salmon populations found discordant phenotypic females possessed one or two full genomic copies of sdY, but these copies were localised to autosomes (Ayllon et al., 2020). If autosomal sdY is also present in the Tasmanian Atlantic Salmon population, then concordant phenotypic males with multiple sdY copies should also be evident (Ayllon et al., 2020). Copy number estimates of such individuals would be expected to appear as high value outliers. In this study, we found no individuals with an atypical high sdY copy number. In addition, the present study found at least one individual with a reduced *sdY* copy number, possibly indicating *sdY* mosaicism (Brown et al., 2020).

Further Development

In order to develop an effective protocol involving aromatase inhibitors for neo-male production in Atlantic Salmon, the developmental period where gonadal phenotype is sensitive to estrogen manipulation must first be identified. Studies have demonstrated this period is around the time of hatching in *Oncorhynchus* spp., however our findings suggest it is later than this in Atlantic Salmon. This project found that *cyp19a1a*, which encodes the production of the aromatase enzyme and subsequently endogenous estrogens, was upregulated in a female-specific manner from 66 dph. As such, this developmental stage is a promising candidate for the application of aromatase inhibitor treatments. However, it is unclear whether the gene expression detected at this stage would translate to sex-related differences in aromatase enzyme production and estrogen synthesis. Further study is needed to determine at which age female-dominant aromatase enzyme production and circulatory estrogen levels are initiated. This age will be a promising timepoint for the successful application of aromatase inhibitor treatments to induce female-to-male sex reversal. In addition to treatment timing, further refinement of treatment duration and dose may be needed for aromatase inhibitor treatments to be sufficient for high rates of masculinisation in Atlantic Salmon.

Extension and Adoption

The projects results were presented and shared with the Tassal Group throughout the execution of the project. The final report has also been submitted to Tassal, and this will assist in their R&D plans and initiatives. The information in this report will likely lead to the planning of future studies to improve the production of neo-males by Tassal.

Abbreviations

aldh1a2 – aldehyde dehydrogenase 1 family, member A2

amh – anti-Mullerian hormone

cyp19a1a – cytochrome P450, family 19, subfamily A, polypeptide 1a

cyp19a1b – cytochrome P450, family 19, subfamily A, polypeptide 1b

cyp26a1 – cytochrome P450 Family 26 Subfamily A Member 1

cyp26b1 – cytochrome P450 family 26 subfamily B member 1

dat – days after treatment

ddpf – degree days post fertilisation

DMSO – dimethyl sulfoxide

dph – days post hatch

foxl2a – forkhead box L2a

gDNA – genomic DNA

gsdf – gonadal soma derived factor

MDHT – 17α-methyldihydrotestosterone

rspo1 – r-spondin 1

tbx1a – T-Box Transcription Factor 1

sdY – sexually dimorphic on the Y chromosome

Project materials developed

Manuscripts published

Brown, M. S., Evans, B. S., & Afonso, L. O. (2020). Discordance for genotypic sex in phenotypic female Atlantic Salmon (*Salmo salar*) is related to a reduced sdY copy number. *Scientific reports*, *10*(1), 1-10.

Brown, M. S., Evans, B. S., & Afonso, L. O. (2021). Genotypic female Atlantic Salmon (*Salmo salar*) immersed in an exogenous androgen overexpress testicular-related genes and develop as phenotypic males. Aquaculture, accepted 18-07-21. AQUACULTURE-D-21-01638R1

Manuscripts in preparation

Brown, M. S., Evans, B. S., & Afonso, L. O. (2021). Characterisation of gene expression and histological gonadal development during sex differentiation in Atlantic Salmon (*Salmo salar*).

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