Gene expression of the gonadotropin receptors and anti-Müllerian hormone in early maturing male Atlantic salmon parr, *Salmo salar*

Genuttryck av gonadotropinreceptorerna och anti-Müllerian hormon hos tidigt mognande atlantlaxhanar, *Salmo salar*

Degree Project of 30 credit points
Biology

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Serial Number: 08:110
Abstract
An up-regulation of gene expression of the gonadotropin receptors FSHR and LHR, and a down-regulation of anti-Müllerian hormone (AMH), in the gonads of early maturing male Atlantic salmon parr (*Salmo salar*) have been shown to take place during the process of sexual maturation. It has however not been determined if this happens prior to or after the onset of spermatogenesis. The aim of this study was to see if such an up-regulation of the gene expression of FSHR and LHR mRNA, and down-regulation of AMH in the gonad tissue could be seen prior to the onset of spermatogenesis in early maturing salmon. For this study gonad tissues were sampled before and after the onset of spermatogenesis. Small pieces of testis tissue were removed using surgery from individually tagged one year old male Atlantic salmon parr in April prior to the onset of spermatogenesis. The same salmon were sampled again in July and maturing and non-maturing fish could be distinguished by differences in GSI. Each tissue sample from April could be matched with the July sample from the same individual by pit-tag numbers. This made it possible to separate the April samples into a maturing and non-maturing group. Gene expression levels were analysed using real-time PCR. The findings of this study showed that all three genes were expressed in the gonads in April but no significant difference in expression levels between maturing and non-maturing salmon was seen for any of the genes, which indicate that no up- or down-regulations had taken place in early maturing fish at this time. In July however, total FSHR and LHR expression levels/testis were significantly higher in maturing salmon which is in accordance with previous studies. AMH expression levels/unit RNA in July were found to be on average 25 times higher in the non-maturing group. A 100-fold drop in AMH from April through July was seen in the maturing fish, while only a 4-fold drop was seen in the non-maturing group which may indicate that a down-regulation of AMH expression took place as spermatogenesis was initiated in the maturing males.

Sammanfattning
En uppreglering av genuttrycket av gonadotropinreceptorerna (FSHR och LHR) samt en nedreglering av anti-Müllerian hormon (AMH) har observerats i gonaderna under spermatogenezien hos tidigt mognande atlantlaxhanar (*Salmo salar*). Man har dock inte kunnat visa huruvida detta sker före eller efter spermatogenezien inletts. Syftet med denna studie var att undersöka om en uppreglering av FSHR och LHR samt en nedreglering av AMH kunde ses hos tidigt mognande laxar redan före spermatogenesen inletts. I den här studien användes gonadprover tagna före och efter spermatogenesen inletts. Små gonadvävnadsprover togs från individuellt märkta ettåriga atlantlaxhanar i april, före spermatogenesen inletts, genom ett operativt ingrepp. I juli togs sedan gonadprover från samma individer igen och mognande och icke-mognande individer kunde nu särskiljas genom deras GSI-värden. Varje gonadprov från april kunde matchas med proverna från juli för samtliga individer genom att jämföra pit-tag nummer och möjliggjorde att även aprilproverna kunde sorteras i en mognande och en icke-mognande grupp. Nivåerna av genuttryck av mRNA analyserades med real-time PCR. Resultaten från denna studie visade att alla tre generna var uttryckta i de omogna gonaderna i april men det var ingen signifikant skillnad mellan de mognande och icke-mognande fiskarna för någon av generna, vilket betyder att det ännu inte skett någon upp- eller nedreglering vid denna tidpunkt hos de tidigt mognande fiskarna. I juli var dock de totala FSHR och LHR mRNA nivåerna/testikel signifikant högre hos mognande fiskar vilket överensstämmer med tidigare studier. AMH mRNA/enhet RNA var uttryckt 25 gånger högre i de omogna gonaderna jämfört med de mognande. Mellan april och juli föll AMH nivåerna hos mognande fiskar nästan 100 gånger, medan de hos de omogna minskade endast 4 gånger vilket kan indikera att en nedreglering av genuttrycket för AMH skett i de individer där spermatogenesen inletts.
Introduction

Puberty is a developmental event characterized by the transition of an immature juvenile to that of a mature adult capable of reproducing sexually. In teleost fish, as well as in mammals and other vertebrates, puberty is under the control of the brain-pituitary-gonad axis (BPG axis), which is activated only upon the onset of puberty (Schulz and Goos, 1999; Adams et al., 2002). The BPG axis is in turn activated as a result of a number of external and internal factors such as temperature, photoperiod and growth rate. Sexual maturity in male teleost fish is initiated by spermatogenesis and reaches completion with the event of spermiation, a period during which dramatic changes in steroid hormone production and in the size and cellular composition of the testes takes place (Blázquez et al., 1998). The process is divided into five different stages that are based on the most advanced germ cell type found within the testes (Grier, 1981; Schulz, 1984); from the immature testes with almost exclusively primary spermatogonia to the spermiating testes with the presence of predominantly spermatozoa. In early maturing Atlantic salmon (Salmo salar) spermatogenesis is initiated in late May and spermiation takes place in October-November (Maugars and Schmitz, 2008a). Male Atlantic salmon can adopt two different life-strategies (Fleming, 1998). They can either migrate from freshwater out to sea after their first or second year and spends several years there, increasing in body mass, after which they return to their native rivers to spawn. A second strategy is to stay in the freshwater and commit to sexual maturation already as a one or two year old parr. The factors that determine whether or not an individual will commit to early sexual maturation and the endocrine regulation of the process are still not fully understood.

Physiological indicators of commitment to early sexual maturation in salmonids are the plasma and pituitary levels of gonadotropin hormones and their transcript levels. There are two gonadotropins found in teleost fish (Swanson et al., 1991): follicular stimulating hormone (FSH) and luteinizing hormone (LH), which are both produced and secreted by the pituitary gland. Their secretion is under the stimulatory influence of the brains output of gonadotropin releasing hormone (GnRH) (Blázquez et al., 1998). FSH and LH act at the gonadal level by binding to their specific cell surface receptors, the FSH receptor (FSHR) and the LH receptor (LHR), located almost exclusively in the gonads (Oba et al., 2001; Kwok et al., 2005). They thereby regulate the production of the sex steroids like testosterone and 11-ketotestosterone, by controlling gene expression of the enzymes that are involved in their synthesis (Planas and Swanson, 1995). In Atlantic salmon it has been shown that pituitary FSH transcripts levels start to increase in May, before any signs of developmental changes in the testes associated with sexual maturation (Maugars and Schmitz, 2008a) and since FSH stimulates spermatogonial proliferation and steroidogenesis it is thereby believed to be involved in the onset of spermatogenesis (Swanson et al., 1989; Loir, 1999). Pituitary LH transcript levels, on the other hand, peak just before spermiation and has therefore been suggested to play a crucial roll in the final steps of maturation. Similar expression profiles have been found also in other salmonid species during the process of sexual maturation (Prat et al., 1996; Gomez et al., 1999; Swanson et al., 1999). Gonad expression levels for FSHR and LHR in Atlantic salmon, on the other hand, have a somewhat different temporal profile compared to their cognate hormones. Gonad mRNA levels of both receptors has been shown to increase significantly in the beginning of spermatogenesis and continued to increase during mid-spermatogenesis (Maugars and Schmitz, 2008a). In maturing males LHR further continued to increase until spermiation while FSHR fluctuated at a significantly higher level then that for immature individuals. FSHR levels then declined during spermiation while LHR peaked. This corresponds to findings that the testes are more sensitive to the action of LH during the late stages of spermatogenesis while their sensitivity to FSH diminishes (Planas and Swansson,
Studies on different teleost species have shown that FSHR is exclusively expressed on Sertoli cells in the gonad tissue while LHR has been found only on Leydig cells (Yan et al., 1992; Miwa et al., 1994; Oba et al., 2001), even though studies on coho salmon could not rule out the possible presence of FSHR on Leydig cells (Yan et al., 1992). The proliferation of Leydig cells in the coho salmon testes during spermatogenesis was shown to be correlated with the increase of mRNA expression levels of LHR in the gonads. FSHR binds both gonadotropins, but with a higher affinity for FSH, while LHR has been shown to bind only LH (Yan et al., 1992; Miwa et al., 1994). The same binding preferences by FSHR and LHR have been demonstrated for other teleost species as well (Kwok et al., 2005; Vischer et al., 2004). It is important to note, however, that an increase in FSHR and LHR mRNA does not necessarily mean that functional gonadotropin receptors are actually expressed on the cell surfaces in relation to these levels. LHR was shown to only be expressed on the cell surface of Leydig cells during the last stage of testicular maturation in coho salmon (Miwa et al., 1994), while LHR mRNA expression levels of Atlantic salmon started increasing already during spermatogenesis (Maugars and Schmitz, 2008a), which could indicate that there might not be such an obvious correlation between the two. The regulation of the gonadotropin receptors in teleost fish is still not clearly understood and requires further investigation.

Another hormone believed to play an important role in the initiation of sexual maturation in teleost fish is a mammalian homolog of anti-Müllerian hormone (AMH) (Miura et al., 2002). In mammals it serves several functions during the process of sexual differentiation and puberty (Wu et al., 2004); it is crucial for testosterone production as well as Leydig cell development and inhibits these processes if not down-regulated in a normal manner. Little is known about the effects of AMH during sexual maturation in fish. However, in male Japanese eel (Anguilla japonica) it has been found to inhibit entrance into spermatogenesis when present at high levels in the testes and when down-regulated spermatogenesis is initiated (Miura et al., 2002). It has recently been suggested that the expression of AMH mRNA in the gonads of maturing male Atlantic salmon is down-regulated, while remaining high in the males that stay immature (Maugars and Schmitz, 2008b).

As mentioned above, the gene expression of FSHR and LHR has been shown to be up-regulated and AMH down-regulated in the gonads of male Atlantic salmon during the beginning of spermatogenesis. However, it has not been determined whether or not this regulation starts prior to or after the initiation of sexual maturation due to the fact that so far it has been impossible to distinguish males that commit into sexual maturation from those that stay immature prior to testis differentiation. The aim of this study was to investigate if such an up-regulation in mRNA expression levels of LHR and FSHR, and/or a down regulation of AMH in the testes in early maturing male Atlantic salmon parr take place before sexual maturation is initiated. Gonad tissue samples used in this study came from one year old male Atlantic salmon that had been collected at two different sampling dates, in April prior to the onset of spermatogenesis and a second sampling in July after spermatogenesis had been initiated. What made this study possible was the way by which the gonad tissue had been collected using a surgical method that allowed the fish to survive the first sampling. The fish had then been kept alive until the second sampling in July when another set of gonad tissue samples were extracted from the intact testis of the same individuals. At this time maturing fish could be distinguished from non-maturing fish by their gonadosomatic index (GSI) and sorted into a maturing and a non-maturing group. All fish were individually tagged and matching of the pit-tag numbers for the two sampling dates made it possible to divide the April samples into a maturing and a non-maturing group also. As well as analysing expression levels in April, a comparison of mRNA expression levels of the genes was also made between
the maturing and non-maturing individuals for the sampling in July where maturing salmon were expected to show a higher expression level of FSHR and LHR, and non-maturing fish a higher expression of AMH.

Materials and methods

Fish and sampling methods
The fish used in this experiment were 1-year old male Atlantic salmon parr that were kept under normal hatchery rearing conditions at the Swedish Board of Fisheries research station at Älvkarleby, Sweden. Fish gonad tissue samples were obtained by Professor Bertil Borg (Stockholm University). The first fish sampling took place on April 11th-13th, 2007. The fish were anaesthetized using 2-phenoxyethanol and subsequently weighed. An abdominal cut was placed medially, whereby a piece of one of the testis was surgically removed and placed in 100 µl RNAlater. A pit tag was inserted into the abdominal cavity of each individual after which the cut was sutured. The fish were then transferred to fresh water where they regained consciousness. They were then kept under standard hatchery rearing conditions until the second sampling which took place on July 19th, 2007. The fish were then killed by administering an overdose of 2-phenoxyethanol. They were weighed and the previously unsampled testis were removed and also weighed. A piece of the mid section of the testis of each individual were then surgically removed, placed in 200 µl RNAlater and stored at -80°C. Pit-tag numbers were scanned to be able to match individuals from the two sampling dates. Animal treatment was approved by the ethics committee of the Swedish National Board for Laboratory Animals. A total of 29 fish were operated and sampled in April of which one died before the second sampling in July. In July 16 of the 28 individuals remained immature and 12 had started maturing. Identifying the fish that had started maturing in July made it possible to match their pit-tag numbers with the samples from April and thereby sort the April-samples into a non-maturing and maturing group. Salmon that started maturing during the experiment was determined by the gonadosomatic indices (GSI=gonad weight/body weight*100) calculated for the July sampling. A selection of 10 fish that had started maturing during the course of the experiment and 10 fish that remained immature was made for this study. A total number of 20 fish were used, for a sum of 40 gonad tissue samples (20 from April and 20 from July). Two of the fish samples from the maturing July group were excluded from the final analysis due to irregular values obtained for the reference gene (see Results).

Quantification of gene expression
RNA extraction
Tissue samples were removed from the RNAlater-solutions and weighed. Samples from April and samples of non-maturing individuals from July were used whole, whereas smaller parts of the samples from mature individuals from July were sliced off and used. Tissue sample weights varied between 0.3-20.3 mg. All work with RNA was performed on ice unless stated differently. The samples were then transferred to RNase free tubes, each containing 200 µl Trizol reagent (Invitrogen) for total RNA extraction according to the manufacturer’s instructions. The tissue samples were homogenized using a tissue grinder and incubated at room temperature for approximately 5 minutes after which 40 µl of chloroform was added to each tube. The content of each tube was then mixed by vigorous shaking and incubated for an additional 5-10 minutes at room temperature. The samples were subsequently centrifuged at 13 000 n/min at 4°C for 20 minutes. The colorless aqueous upper phase of each sample, containing the RNA, was then transferred to a new RNase free tube, to which 1 µl of glycoblu and 100 µl of cold (-20°C) isopropanol were added. The samples were then
vortexed and placed at -20°C for 30 minutes or left overnight after which they were centrifuged for 20 minutes at 14,000 n/min at +4°C. This rendered a small blue pellet, containing the extracted RNA, in the bottom of each tube. The pellets were washed by first removing the supernatant and then adding 200 µl of cold (-20°C) 75% ethanol to each tube. The tubes were then centrifuged at 7500 x g at +4°C for 5 minutes and stored at -20°C until further DNase treatment.

**DNase treatment**
The samples were first centrifuged at 7500 x g at +4°C for 5 minutes after which the ethanol was removed and the pellets were left to dry for 5-10 minutes in the tubes. The pellets were dissolved by adding 10 µl of nuclease free water (NF water) and pipetting up and down. 1.1 µl of TURBO DNase buffer and 1.0 µl of TURBO DNase, were added and samples were mixed and incubated at 37°C for 30 minutes. After incubation, 1.1 µl of DNase Inactivation Reagent was added to each sample and left in room temperature for two minutes while mixing occasionally. The samples were then centrifuged at 10,000 n/min at +4°C for 2 minutes after which the supernatant was transferred to new labelled strips and stored at -80°C.

**Total RNA concentration measurements**
The total RNA concentration for each sample was measured using the Quant-it™ RiboGreen® RNA Reagent and Kit on the MX3000P® PCR machine (Stratagene) using the MxPro™ QPCR Software (Stratagene) for measuring concentrations. Dilution series (low range and high range) for the construction of a standard curve were made from a 100 ng/µl RNA stock solution according to the manufactures recommendations. The low range standard curve was used for the measurements and consisted of the following concentrations: 5, 4, 3, 2, 1, 0.5 and 0.1 ng RNA/µl. 1 µl of each sample was then diluted either 1:100 or 1:200 by adding NF water. A master mix containing a ratio of 66 µl of 20 X TE, 0.33 µl of RiboGreen reagent and 593.7 µl of NF water was prepared and 45 µl of the mix was added to each well on a 96-well plate. The samples and the standards were then added to the wells in volumes of 5 µl/well for a total volume of 50 µl/well. The wells were subsequently covered with optical strip caps. All samples and standards were run in duplicates. The plate was vortexed for 3 minutes before being placed in the PCR machine. Absorbance values were measured at a wavelength of 260 nm at 25°C and RNA concentrations/µl sample were obtained from the software. Total RNA concentrations for each sample were then calculated by correcting for the dilution factor by multiplying the obtained concentration with 100 or 200, depending on which dilution was used.

**cDNA synthesis**
100 ng of total RNA were reverse transcribed to synthesise cDNA. Each RNA sample was mixed with 1 µl of random primers, 1 µl of 10 mM dNTP and NF water for a total volume of 13 µl for each sample. Samples were then placed in a programmable thermal controller and heated to 65°C for five minutes after which they were immediately incubated on ice for at least one minute. The samples were then briefly centrifuged and a RT mix containing 1µl DTT, 1µl RNaseOut (40U/µl), 4µl 5x First-strand Buffer and 1µl Superscript III RT (200U/µl) was added to each tube and the contents were carefully mixed by gently pipetting up and down. The RT reaction was carried out on the programmable thermal controller which was programmed for 25°C for 10 minutes, 50°C for 60 minutes and 70°C for 15 minutes. Samples were stored at -20°C.
Real-time PCR
When using real-time PCR as a tool to measure relative amounts of mRNA for a target gene, which was done in this study, normalization of data using an internal reference gene, also called a housekeeping gene, is often necessary. This is due to possible differences in RNA loading when using reverse transcriptase (RT) (Olsvik et al. 2005), which would lead to different amounts of starting material in the samples used in the real-time PCR assays. A good reference gene should be expressed at a constant level independent of developmental state in the studied tissue. By quantification of such a gene for a sample set it can indicate if the amount of RNA that was reversely transcribed differed between samples. This potential discrepancy can be adjusted for by dividing the measured relative concentrations mRNA for a gene of interest with the concentration for the reference gene. 18s ribosomal RNA (18s rRNA) is often used as a housekeeping gene for normalization of real-time PCR data due to its constant and high expression levels in different tissues and developmental stages (Kusakabe, 2006,) and was therefore chosen as the reference gene used in this study. However, the use of 18s rRNA as one of the best reference genes has been challenged (Olsvik et al., 2005; Ingerslev et al., 2005) and other genes have been proposed as being more stably expressed and therefore more reliable. Another somewhat commonly used reference gene is elongation factor 1Aa (EF1Aa) and a comparison between EF1Aa and 18s rRNA was included here to test the stability of the selected reference gene and whether EF1Aa would be a better choice for data normalization.

Real-time PCR assays for FSHR, LHR, AMH, 18s RNA and EF1Aa were performed using the SybrGreen fluorescence dye Mesa Green and a Stratagene Mx3000P® real-time PCR machine. A standard curve dilution series was prepared from a standard cDNA preparation made from salmon gonads at different developmental stages and was used for all real-time PCR assays and every plate set-up. The standard curve dilutions used for the genes were as follows: FSHR 1:100-1:1600, LHR 1:50-1:1600, AMH 1:100-6400, 18s RNA 1:100-3200 and EF1Aa 1:10-1:800. cDNA samples were diluted 1:10 for FSHR and LHR, and 1:100 for AMH, EF1Aa and 18s rRNA. All samples and the standard curve were run in duplicates. For each PCR assay, samples, standard and reaction mix were applied to 12 strips, for a total of 96 wells and each real-time PCR reaction contained a total of 15 µl PCR mix, consisting of 1 µl cDNA template, 0.3 or 0.45 µl of 10 mM reverse primer and forward primer, 7.5 µl of SybrGreen Mix and 5.9 or 5.6 µl of NF water. Primer concentrations (for both forward and reversed) were 200 nM for 18s RNA and LHR, and 300 nM for EF1Aa, FSHR and AMH. For primer nucleotide sequences used, see Table 1. All strips were covered with optical plastic caps and placed in the PCR machine. The thermal cycling conditions were 95°C for 10 min followed by 34-40 cycles (34 for 18s rRNA and 40 for all other genes) at 95°C for 30s, 58-60°C (58°C for AMH and 60°C for all other genes) for 1 min, and 72°C for 30s. The amplified product specificity was verified by running melting curves at the end of the PCR cycles. R² values >0.997 were obtained for all standard curves and efficiencies varied between 80.2-100.2 % with the exception of LHR where the efficiency was only 54.6%.
Table 1. Forward (FW) and reverse (RV) primer nucleotide sequences for FSHR, LHR, AMH, 18s RNA and EF1Aa used in the real-time PCR assays.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Direction</th>
<th>Sequence (5'→3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSHR</td>
<td>FW</td>
<td>TTCAGGGGTGAGGGGTTAAA</td>
</tr>
<tr>
<td></td>
<td>RV</td>
<td>CAGATGTTTTGACCTTGTTGATGG</td>
</tr>
<tr>
<td>LHR</td>
<td>FW</td>
<td>CCTGAGAAGAGTCCAGCATATAGA</td>
</tr>
<tr>
<td></td>
<td>RV</td>
<td>GAAGATTTCATTGAGGTCGAGAAG</td>
</tr>
<tr>
<td>AMH</td>
<td>FW</td>
<td>CAAAAACACCAGAGACAGGACAA</td>
</tr>
<tr>
<td></td>
<td>RV</td>
<td>TATCCGTTGAGAAAGCACCA</td>
</tr>
<tr>
<td>18s RNA</td>
<td>FW</td>
<td>CTCAACACGGGAAACCTCAC</td>
</tr>
<tr>
<td></td>
<td>RV</td>
<td>AGACAAAATCGCTCCACCAAC</td>
</tr>
<tr>
<td>EF1Aa</td>
<td>FW</td>
<td>GAGAACCATTGAGAAGTTCGAGAAG</td>
</tr>
<tr>
<td></td>
<td>RV</td>
<td>GCACCCAGGCATACTTGAAAG</td>
</tr>
</tbody>
</table>

Data analysis

Relative gene expression values were calculated by the MxPro™ QPCR Software (Stratagene) and were then adjusted for dilution by multiplying by 10 or 100, depending on the gene. FSHR, LHR and AMH expression quantities were then normalized by division with corresponding sample concentrations measured for 18s RNA. Expression levels for the April samples are presented as relative mRNA levels/100 ng total RNA. Further normalization of data was necessary for the July sampling due to the difference in gonad size of the different groups, where the gonads in the mature fish had an average testis weight 98 times bigger than that of the non-maturing fish. This causes what is referred to as a “dilution effect” which is due to changes in cell distribution and density as the testis grow. Relative mRNA amounts will therefore appear to be lower then they are in the mature testis. The following equation suggested by Kusakabe et al. (2006) was used to correct for this:

\[
\text{Target mRNA level} = \frac{\text{PCR target gene quantity}}{\text{PCR reference gene quantity}} \times \frac{\text{amount of extracted RNA}}{\text{tissue weight for RNA extraction}} \times \frac{\text{gonad weight}}{\text{body weight}}
\]

The calculated amount of target gene mRNA was then expressed as total RNA/testis. July samples are presented both as relative mRNA levels/100 ng total RNA but also as corrected according to the above formula. A student’s t-test was used to check for significant differences in average mRNA expression levels for the different target genes between the maturing and non-maturing groups at the different sampling occasions. The accepted level of significance was p<0.05. Data are presented as means of relative mRNA expression levels ± standard error (S.E.). For comparison of the two reference genes, coefficients of variation were calculated and a linear regression analysis was also performed and used to calculate residuals.
Results

Changes in body weight and GSI
Non-maturing salmon weighed on average twice as much as the maturing salmon in April (see Table 2), which was a significant difference (t-test, p<0.001). From April through July, both maturing and non-maturing individuals had increased their body weight and the non-maturing salmon remained on average significantly heavier than the maturing individuals (t-test, p<0.05). The difference in GSI (%) for July was significantly higher in maturing individuals (t-test, p<0.001), being on average almost 140 times that of the non-maturing fish.

Table 2. Body weight of male Atlantic salmon parr at the two sampling occasions. N = number of samples. GSI (%) values shown for the July sampling groups. Data shown as means (± S.E). Level of significance **=p<0.05, ***=p<0.001.

<table>
<thead>
<tr>
<th>Month</th>
<th>Developmental stage</th>
<th>N</th>
<th>Mean weight (g)</th>
<th>Mean GSI (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>April</td>
<td>Maturing</td>
<td>10</td>
<td>8.01 (1.15)</td>
<td>n.d</td>
</tr>
<tr>
<td></td>
<td>Non-maturing</td>
<td>10</td>
<td>16.1 (0.88) ***</td>
<td>n.d</td>
</tr>
<tr>
<td>July</td>
<td>Maturing</td>
<td>8</td>
<td>50.0 (8.42)</td>
<td>4.04 (1.27) ***</td>
</tr>
<tr>
<td></td>
<td>Non-maturing</td>
<td>10</td>
<td>69.3 (1.86) **</td>
<td>0.029 (0.0011)</td>
</tr>
</tbody>
</table>

Total RNA amounts
A 34 times higher mean amount of total RNA (µg RNA/g body weight) was measured in maturing salmon in July. On average more than four times more total RNA was extracted/g tissue from immature testes than for maturing testes in July (data not shown). No such difference in total amount of extracted RNA/g tissue was observed between the maturing and non-maturing groups in April.

Comparison of 18s rRNA and EF1Aa mRNA
The comparison between the reference genes 18s rRNA and EF1A was carried out to determine which of the two showed the greatest transcriptional stability, i.e. it should be expressed at a constant level at any given time in the tissue that is being used, since this is an important factor when normalizing mRNA expression data for the target genes. The coefficient of variation (CV) was 62.5 % and 45.0 % of the mean for all samples (n=38) for EF1Aa and 18s rRNA respectively. The CV for 18s RNA was hence 1.4 times lower than that of EF1A, 18s rRNA therefore showing a greater transcriptional stability. The calculated residuals for the relative RNA transcription levels of each sample are presented in Fig. 1, illustrating the smaller variation between samples for 18s rRNA. Two samples from the July sampling date were excluded from the final analysis due to obtained transcript levels that were too high compared to the other samples and therefore considered unreliable (data not shown).
Fig. 1 Residual plot for 18s rRNA and EF1A mRNA from both sampling dates of male Atlantic salmon parr. Sample 1-20 correspond to samples from April and sample 21-38 from the July sampling. Open circles represent 18s RNA and filled diamonds represent EF1A.

Gene expression levels

FSHR
There was no significant difference in expression of FSHR between maturing and non-maturing salmon in April (Fig. 2a). In July the FSHR expression levels for both the maturing and the non-maturing fish had dropped when looking at the relative FSHR level/100 ng total RNA (Fig. 2a). Levels were higher, but not significantly, for non-maturing salmon in July. However, when normalizing the July FSHR expression levels for total RNA yield/testis and body weight as described in Materials and method, a significant difference (t-test, p<0.001) between maturing and non-maturing salmon was observed, the maturing fish showing a 17-fold higher FSHR expression level (Fig. 3).

LHR
Relative levels of LHR mRNA were slightly higher for the maturing group in April but the difference was not significant (Fig. 2b). The non-maturing individuals had a higher expression level of LHR in July when measured/100 ng total RNA, but again the difference was not significant (Fig. 2b). In July, both the maturing and non-maturing salmon had lower expression levels of LHR mRNA/unit RNA compared to levels in April. When transcript levels were adjusted for total RNA yield/testis and body weight the maturing group showed an almost 23 times higher transcript level compared to the non-maturing group (Fig. 3) and the difference between the groups did become significant (t-test, p<0.001).

AMH
Relative expression levels of AMH were approximately the same for both groups in April (Fig. 2c). In July, however, a significant difference between the maturing and non-maturing group was seen (t-test, p<0.001) when mRNA levels were measured/100 ng RNA. The non-maturing salmon showed 25 times higher expression levels than the maturing group. AMH levels/100 ng total RNA for the maturing salmon had decreased a 100-fold from April through July, while the levels for the non-maturing fish only showed a 4-fold decrease. When adjusting for total RNA yield/testis and body weight no significant difference was observed and AMH levels were somewhat higher in the maturing males (Fig. 3).
Fig. 2 Relative mRNA levels for a) FSHR, b) LHR and c) AMH from the April and July sampling of maturing (n(April)=10 and n(July)=8) and non-maturing (n=10 for April and July) male Atlantic salmon parr. Data is shown as mean ± S.E for each group. Level of significance *** = p<0.001. Maturing salmon is represented by grey bars and non-maturing by black bars. Note the scale differences of the y-axis between the graphs.
Fig. 3 Relative mRNA levels for FSHR, LHR and AMH for the July sampling of maturing and non-maturing male Atlantic salmon parr. Data shown as means ±S.E. mRNA levels are expressed as relative levels of total mRNA/testis. Maturing salmon is shown as grey bars and non-maturing as black bars. Level of significance *** = p<0.001.

Discussion

All the relative mRNA levels obtained from the real-time PCR assays for the genes analysed in this study were normalized using a reference gene. Since it is possible that the starting material used in the real-time PCR assays differ between samples due to calculation and/or pipetting errors, a reference gene can be used to verify that the amount is the same and if differences do exist it can be corrected for. Reference gene stability is therefore crucial but expression levels of these genes have been known to sometimes be affected by developmental changes in the tissue being investigated (Kusakabe, 2002). This is undesirable because when the expression levels of the target genes are normalized for the reference gene the calculated transcript levels will not be reflected correctly if the reference gene is also regulated. The reference gene selected for the normalization of data for all genes analysed in this study was 18s rRNA. The result from the real-time PCR assays for 18s rRNA and EF1Aa revealed 18s rRNA as being the more stable of the two, with 1.4 times lower coefficient of variation for the RNA expression levels. In some studies 18s RNA has been found to be more variable in a number of tissues than other commonly used reference genes (Ingerslev et al., 2005; Olsvik et al., 2005) and in one study EF1Aa was found to be the most stable housekeeping gene in Atlantic salmon (Olsvik et al., 2005). A recent reference gene evaluation study by Jorgensen et al. (2006) found on the other hand both 18s rRNA and EF1Aa to be the genes with the highest transcriptional stability out of several frequently used genes. None of these studied however included gonad tissue as one of the tissues analysed. Even though conflicting results are found in regards to the reliability of 18s rRNA, it was estimated, based on the obtained results, as being the better choice for this study.

The result from the comparison of mRNA levels of FSHR and LHR in April between the maturing and the non-maturing fish showed that both receptors were expressed, FSHR levels 2.4 times higher than LHR, but no significant difference existed for either receptor between the groups. This point to the possibility that an up-regulation of these genes prior to the onset of spermatogenesis does not take place, or at least not as early as the date of the first sampling in this study. However, it can not be ruled out that an up-regulation of FSHR and LHR could
take place before spermatogenesis is entered into and would require further studies including sampling dates between mid April and late May as well as sampling of the same individuals when commitment to sexual maturation is measurable and hence allows for the identification of maturing individuals at the earlier sampling dates, as was done in this study. The findings here are however in agreement with a recent study on Atlantic salmon by Maugars and Schmitz (2008a), where transcript levels of FSHR and LHR did not allow for the distinction of maturing and non-maturing individuals prior to the onset of spermatogenesis. It was also shown that the up-regulation of FSHR happens in conjunction with increased pituitary mRNA expression levels of FSH. LHR levels started to increase at the same time while LH levels were still relatively low (but significantly higher than in non-maturing individuals). This could indicate that the gonadotropins may directly or indirectly induce the up-regulation of their respective receptors. Studies on mammals have pointed to the existence of this regulation mechanism for FSH (Maguire et al., 1997). Another possibility is that the increase in FSH and LHR might be a result of the proliferation of Sertoli cells and Leydig cells that takes place during spermatogenesis and not as a result of an actual up-regulation in transcription levels (Walker and Cheng, 2005; Maugars and Schmitz, 2008a). If this is the case then elevated levels of the gonadotropin receptors are unlikely to occur prior to the onset of testicular development since cell proliferation does not take place before this event. For the July samples, when FSHR and LHR expression levels were adjusted only for the reference gene, a decrease in transcript levels for both maturing and non-maturing salmon was observed. This is likely due to a dilution of Leydig and Sertoli cells in the tissue as the testes grow and changes their cell composition (Kusakabe et al., 2006) and does not correctly describe transcript levels. Basing the analysis instead on the total RNA/testis was necessary to make an assessment of the relative expression levels of these genes. After doing this, significant differences were seen for FSHR as well as for LHR between the maturing and non-maturing males showing that an up-regulation of both receptors may have occurred. This is supported by several studies that have found that an up-regulation of transcription levels for both receptor genes in Atlantic salmon, as well as other teleost species, take place during early spermatogenesis (Kusakabe et al., 2006; Maugars and Schmitz, 2008a), a period which for Atlantic salmon spans approximately from late June through mid July.

AMH expression levels in both maturing and non-maturing salmon in April were high compared to FSHR and LHR. No significant difference in AMH transcript levels was seen between the groups. It therefore seems unlikely that any changes in the regulation of this gene have happened at this point prior to the initiation of spermatogenesis. The same has been found in Atlantic salmon in a previous study (Maugars and Schmitz, 2008b), where no difference in gonad AMH expression levels could be seen to allow for the distinction of maturing or non-maturing males at this point. In July, the result for the AMH levels per unit RNA shows that expression levels are higher in the immature testes. This is in accordance with previous studies on teleost fish (Miura et al., 2002; Maugars and Schmitz, 2008b) and it has been suggested that testis AMH production in maturing individuals is down-regulated. The result of this study shows a substantial difference in AMH levels between the maturing group in April compared to July, where July AMH levels/100 ng RNA were a 100 times lower than they were in April. A much smaller four-fold drop in AMH levels between April and July for the non-maturing group was also observed. The drop in non-maturing salmon may be accounted for by the fact that as the fish grows, even though it doesn’t mature, the testes will still grow in proportion to the body mass. During April through May a 4.2-fold mean weight increase for the non-maturing group was observed, which corresponds well with the observed drop in AMH levels. It could be suggested that the number of Sertoli cells that produce AMH in the immature testis remains the same in July as in April if no cell
proliferation has taken place. The cells would therefore be less concentrated in the tissue because of the growth of the testes and cell abundance will therefore be lower/unit tissue. The same reasoning could be used to explain why AMH levels in maturing individuals has drastically decreased in July since their gonads have increased even more in size, weighing on average almost a 100 times more than the immature testis. However, this does not take into account that a proliferation of AMH-producing Sertoli cells in the maturing testes have taken place since testicular maturation has been initiated (Schulz et al. 2005), which would mean that the density of these cells have increased and hence AMH production levels would have dropped less than what was observed. AMH levels could therefore have been expected to be higher and this leads to a possible conclusion that AMH transcription has indeed been down-regulated. Miura et al. (2002) showed in a study on Japanese eel that AMH was expressed in Sertoli cells in immature testes and that, not only did a down-regulation take place, but the gene seemed to have been switched off as spermatogonial proliferation started. It was therefore proposed that AMH was the key factor preventing the onset of spermatogenesis and that may be the case for Atlantic salmon as well although that is yet to be proven. When looking at total AMH RNA/testis, AMH expression levels were instead higher in the maturing group compared to the non-maturing group. This result could maybe be accounted for because testis size of the maturing fish is bigger and could therefore contain more total amount of AMH than a small immature testes whether or not the AMH transcription has been down-regulated. If transcription has been down-regulated but there are more Sertoli cells in the maturing testis, due to Sertoli cell proliferation, the individual cells would show a decreased amount of AMH mRNA production compared to cells in the immature testis but the total amount in the whole testis of the maturing salmon could still be higher because there are more cells that produce it. Even though this might explain the higher mRNA AMH levels in maturing salmon in this study it is still not in accordance with the previous study on AMH in Atlantic salmon by Maugars and Schmitz (2008b), where maturing males showed a lower total AMH transcription level than that of non-maturing males in July. In June, however, the Maugars and Schmitz (2008b) study showed that transcript levels were higher in maturing fish and it is possible that the differences between the studies are due to the timing of the sampling and because different strains of Atlantic salmon were used that may have had slightly different timing of the spermatogenesis stages. If the second sampling of this study had taken place later in July it cannot be ruled out that AMH levels would be lower in maturing salmon.

As in all studies a number of factors may have influenced the obtained data affecting the reliability of the results. One of those factors in this study was that extraction of total amount of RNA/mg tissue from the July sampling was 4 times higher for immature gonad samples than for maturing ones. This was probably due to the difference in tissue consistency between the two groups, which led to problems during the tissue homogenisation process, maturing samples being more difficult to homogenise. This could have led to loss of RNA by samples not being fully homogenized and hence RNA extraction became incomplete. The effects of this would be that, when normalizing mRNA levels for total testis amount and body weight, mRNA levels in maturing individuals became lower than what would otherwise have been the case. The observed difference in FSHR and LHR levels between the maturing and the non-maturing group would therefore have been more pronounced. Another problem encountered during the study was a very low efficiency value (54.6 %) for the real-time PCR assay for LHR. The efficiency value is a measurement of how effectively the PCR-product is amplified for each cycle, a 100 % efficiency indicating a doubling of the product for each cycle which is the desirable result. An acceptable and reliable efficiency value should be above 90 % as stated by the manufacturer of the Stratagene Mx3000 PCR machine, so an efficiency value of
54.6 % is quite poor and indicates that the relative mRNA values obtained for LHR is less reliable than what could be wished for. The obtained efficiency value for 18s rRNA was somewhat low as well, reaching only 80.2 %. This was however deemed a good enough result to use for normalization of data for the target genes, although it should to be pointed out that it might have had an effect on the results.

In conclusion, the experimental method used to collect the tissue samples used in this study made it possible to investigate if any differences in gene expression of FSHR, LHR and AMH prior to the beginning of sexual maturation exist. The result of the study showed that there were no significant differences in gene expression of FSHR, LHR or AMH between maturing and non-maturing Atlantic salmon as early as in April, prior to the onset of spermatogenesis, and hence no up- or down-regulation of either one of the genes had yet taken place. Significant differences were however observed, as hypothesised, between the two groups in July; maturing salmon showing higher expression levels of FSHR and LHR and lower AMH levels (when expressed as mRNA level/100ng total RNA), which is in accordance with what has previously been found in other studies. AMH levels, when expressed as total testicular RNA content, where instead found to be higher in maturing males, which could possibly explained by Sertoli cell proliferation and the time of sampling rather than that no down-regulation has taken place. It would be interesting in the future to see studies on the regulatory mechanisms for the gonadotropin receptors and the roll of AMH in Atlantic salmon, to further unveil their functions in the process of spermatogenesis.

Acknowledgements

I would like to thank my supervisor Monika Schmitz for all her support, enthusiasm and for always taking the time to help. I would also like to thank Bertil Borg for obtaining the salmon samples and Maria Malmström for various practical assistance in the lab.

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