Androgens directly stimulate spermatogonial differentiation in juvenile Atlantic salmon (Salmo salar)

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Abstract

We studied the effects of androgens on early stages of spermatogenesis along with androgen receptor binding characteristics and the expression of selected testicular and pituitary genes. To this end, immature Atlantic salmon postsmolts received testosterone (T), adrenosterone (OA, which is converted in vivo into 11-ketotestosterone, 11-KT) or a combination of the two androgens (T + OA). Treatment with OA and T elevated the plasma levels of 11-KT and T, respectively, and co-injection of OA with T lead to high 11-KT levels but prevented plasma T levels to reach the levels observed after injecting T alone. Clear stimulatory effects were recorded as regards pituitary lhb and gnrhr4 transcript levels in fish receiving T, and to a lesser extent in fish receiving OA (but for the lhb transcript only). The two androgen receptors (Ara1 and Ara2) we cloned bound T and 11-KT and responded to these androgens in a similar way. Both androgens down-regulated testicular amh and increased igf3 transcript levels after 1 week of treatment, but effects on growth factor gene expression required sustained androgen stimulation and faded out in the groups with the decreasing T plasma levels. In fish exhibiting a sustained elevation of 11-KT plasma levels (OA and T + OA groups) for 2 weeks, the number of differentiating spermatogonia had increased while the number of undifferentiated spermatogonia decreased. Previous work showed that circulating gonadotropin levels did not increase following androgen treatments of gonad-intact immature male salmonids. Taken together, androgen treatment of immature males modulated testicular growth factor expression that, when sustained for 2 weeks, stimulated differentiation, but not self-renewal, of undifferentiated type A spermatagonia.

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1. Introduction

Pubertal gonad maturation in vertebrates usually requires stimulation by the pituitary gonadotropins luteinizing hormone (Lh).
Fsh is a potent steroidogenic gonadotropin in fish (Chauvigné et al., 2012; García-López et al., 2010, 2009; Planas and Swanson, 1995) and in prepubertal Japanese eel (Anguilla japonica). Fsh-stimulated androgen production mediated the stimulatory effect of Fsh on spermatogenesis (Ohta et al., 2007). However, studies in trout (Oncorhynchus mykiss; Sambroini et al., 2013a) showed that Fsh can also directly change Sertoli cell transcript levels, including transcripts encoding growth factors that modulate germ cell differentiation, such as amh (Skaar et al., 2011; Miura et al., 2002). Moreover, androgens can change testicular mRNA levels coding for proteins potentially relevant for the onset of spermatogenesis (Rolland et al., 2013). Thus, Fsh can directly modulate Sertoli cell functions, or first stimulate Leydig cell androgen production that then modulate spermatogenesis via androgen receptor expressing testicular somatic cells.

The decreases in testicular amh mRNA levels during gonadotropin-induced (Miura et al., 2002) or natural puberty (Maugars and Schmitz, 2008) as well as the fact that Amh blocked androgen-induced spermatogenesis in prepubertal eel (Miura et al., 2002) are interesting to note. In adult zebrafish (Danio rerio), Amh inhibited the differentiation of type A spermatagonia, and moreover inhibited Fsh-stimulated steroidogenesis (Skaar et al., 2011). Members of the insulin-like growth factor (Igf) family, on the other hand, stimulated germ cell proliferation in trout (Loir, 1999), or were required as permissive factor for androgen-stimulated spermatogenesis in eel (Nader et al., 1999). Interestingly, a new member of this family, Igf3, has been described recently (Wang et al., 2008). This gene is predominantly expressed in gonadal tissue and its expression is up-regulated by Fsh in zebrafish (de Waal, 2009; Baudiffier et al., 2012) and in rainbow trout testis (Sambroini et al., 2013a). However, a quantitative evaluation of androgen-induced germ cell development in combination with quantifying testicular mRNA levels of amh and igf3 has not been reported so far.

Thus, in order to evaluate the effects of sex steroids on the initiation of pubertal spermatogenesis, we treated immature male fish with two different androgens. Blood (11-KT and T levels) and testis samples (amh and igf3 transcript levels; quantitative evaluation of germ cell development) were collected for analyses after 1 and 2 weeks. Since sex steroids also modulate pituitary gene expression, we measured fshb, lhb and gnrh4 mRNA levels. Moreover, 11-KT and T had in part similar, in part distinct effects on testicular gene expression patterns in rainbow trout (Rolland et al., 2013). In order to evaluate the biological activities of these androgens via the androgen receptor (Ar), we cloned two androgen receptor subtypes and studied their ligand binding characteristics.

2. Material and methods

2.1. Experimental design

The experiments took place at Matre Research facility (61°N, which is part of the Institute of Marine Research, Bergen (Norway). In a pilot study, different doses of testosterone (Sigma, St. Louis, USA) and adrenosterone (OA; Sigma, St. Louis, USA) were tested on immature Atlantic salmon post-smolts (length 23.6 ± 0.2 cm, body weight 155.5 ± 3.2 grams) in seawater (35 ppt salinity) at a water temperature of 16 °C. To that end, androgens were dissolved in ethanol and then added to 1 part vegetable oil and 1 part shortening (both from Crisco; J.M. Smucker Company, USA), according to Specker et al. (1994). The steroid solution was kept liquid at 25 °C and injected into the body cavity between the pelvic and the anal fin. Only after injecting 25 μg T/g body weight, the highest of three doses tested in the pilot study, increased T plasma levels were recorded after 4 days but had returned to basal levels after 8 days (Fig. 1A). After injection of all doses of OA (1, 5, and 25 μg/g body weight), similarly elevated 11-KT plasma levels were recorded on day 4. Interestingly, increasing doses resulted in an extension of the time period during which elevated 11-KT plasma levels were found but not in higher plasma levels (Fig. 1B). The pilot study also showed that although equal doses were administered, T plasma levels were at maximum only half as high as 11-KT levels. Therefore, for the main experiment, we tripled the dose of T compared to the one of OA. In a previous study on African catfish (Clarias gariepinus), 3-fold higher doses of T than of the 11-oxygenated androgen had to be administered to achieve similar circulating levels (Schulz et al., 2008). Hence, 160 Atlantic salmon postsmolts (length 22.3 ± 1.3 cm, body weight 120.6 ± 22.3 g) were injected with either a fat solution containing no steroid (C), 75 μg T/g of body weight (T75), 25 μg OA/g of body weight (OA25), or a combination of the two androgens (T75 + OA25), establishing four groups with 40 fish each. OA is converted in Atlantic salmon in vivo into 11-KT (Antonopoulou et al., 1999; Borg et al., 1998; Mayer et al., 1990), the main androgen in fish (Borg, 1994). The fish were maintained in four 500 L seawater square tanks under continuous light conditions (at 35 ppt salinity and a water temperature of 12 °C) and fed with commercial feed (Spirit 75, Skretting AS, Stavanger, Norway) distributed by automatic feeders to satiation throughout the whole experimental period.

The experiments have been approved by the local responsible laboratory animal science specialist under the surveillance of the Norwegian Animal Research Authority (NARA) and registered by NARA.
2.2. Sampling

Blood and tissue samples were collected 7 and 14 days after treatment. All fish were anesthetized in seawater containing 10 mg/L of metomidate (Syndel, Victoria, BC, Canada). Body weight and length were determined and blood was collected in heparinized syringes from a caudal vein. After decapitation, the testis were dissected and weighed. One testis was fixed in Bouin’s solution for 24 h and subsequently transferred to 70% ethanol for storage. The other testis and the pituitary were snap-frozen in liquid nitrogen and stored at −80 °C until RNA extraction. Blood was centrifuged and plasma was stored at −80 °C. The gonado-somatic index (GSI) was calculated as: GSI (%) = gonad weight (g) × 100/body weight (g). An initial control sample was not collected since previous work showed that male postsmolts kept under the same photoperiod and temperature conditions remained immature for at least two months (Fjelldal et al., 2011; Melo et al., 2014). Hence, we expected that fish in the control group kept for 2 weeks under these conditions remain immature.

2.3. Measurement of plasma androgens

Individual plasma samples were prepared as described in Melo et al. (2014) and quantification of T and 11-KT by radio-immuno assay (RIA) was carried out as described by Mayer et al. (1990).

2.4. Testis histology and morphometry

Fixed gonads were dehydrated, embedded in paraffin, sectioned at 5 µm and stained with periodic acid Schiff and hematoxylin/eosin to evaluate the progress of spermatogenesis. To this end, ten non-overlapping digital images per fish were randomly taken (Olympus-AX-70; Nikon Digital Camera DXM 1200) at 600× magnification and analyzed quantitatively with the ImageJ freeware (http://image.nih.gov/ij/). Using a grid with 180 intersections, the germ cells under the intersections were identified and counted. The results are expressed as the average number of germ cells counted per microscopic field at 600-fold magnification. We distinguished undifferentiated type A spermatogonia (Aund; single cells with no or very little heterochromatin in the nucleus, one or two prominent nucleoli and a nuclear diameter of ~11 µm; see inset Fig. 3B) and differentiating type A spermatogonia (Adiff; two or more cells in a spermatogenic cyst with nuclei containing some heterochromatin and a nuclear diameter of ~7.5 µm; see inset Fig. 3C) for examples for the morphology of spermatogonia. Further developed germ cell generations (type B spermatogonia, meiotic or post-meiotic stages) were not present in the testis.

2.5. RNA isolation, cDNA synthesis and real-time, quantitative PCR

Total RNA was isolated from frozen tissue (−80 °C) using an RNeasy micro kit (pituities) or RNeasy mini kit (testis) (Qiagen) according to the manufacturer’s instructions. Homogenization of the tissue was performed using 2 ml tubes containing 1 ml lysis buffer (from the RNeasy kit) and zirconium oxide beads in a PrecellysW24 Homogenizer (Bertin, Villeurbanne, France). The amount of beads was 250 mg for pituitaries and 600 mg for gonads. The gonads were DNase treated (according to the RNeasy mini handbook).

RNA quantity and quality were determined by UV absorbance at 230, 260 and 280 nm using a NanoDropWNP-1000 spectrophotometer (NanoDrop technologies, Wilmington, DE, USA). All samples had a 260/280 nm absorbance ratio in the range of 1.7–2.1. Next, cDNA was prepared using 500 ng of each total RNA sample using Superscript VILO cDNA synthesis (Invitrogen, Carlsbad, Germany), as described previously (Melo et al., 2014). The relative salmon gnrhr4, lhb and fshb mRNA levels were quantified in the pituitary as described in Melo et al. (2014). The primers used for amplification and measurement of igf3 and amh mRNA in testes samples were designed using Primer Express 3.0 (Applied Biosystems, Foster City, CA, USA), validated and are listed in Table 1.

TaqMan PCR assays were performed in duplicate, using 384-well optical plates on an ABI Prism Fast 7900HT Sequence Detection System (Applied Biosystems) using the following settings (50 °C for 2 min, 95 °C for 20 s, followed by 40 cycles of 95 °C for 1 s and 60 °C for 20 s). For each 10 µl PCR reaction 2 µl 20-fold diluted cDNA was mixed with 200 nM fluorogenic probe, 900 nM sense primer, 900 nM antisense primer in 1× TaqMan Fast Advanced PCR Master Mix (Applied Biosystems). For each PCR plate, controls without template were run for each gene. Gene expression data were calibrated to the control group, using the ΔΔCT method, as described in detail previously (Rogerd et al., 2001; Good et al., 2012), using ef1α TaqMan assay as endogenous control (Andersson et al., 2009).

2.6. Cloning and pharmacological characterization of androgen receptor (ar) subtypes

Total RNA was extracted from testis tissue and poly(A)-rich RNA was isolated. This material was used to prepare 5’-RACE-ready and 3’-RACE-ready cDNAs, as described by Andersson et al. (2009). For the present RACE protocol, to obtain the full-length Atlantic salmon ar sequences, forward and reverse primers (named 1432–1435; see Table 2) were designed based on a previously obtained, partial ar cDNA (accession number AY049957). Sequencing of the RACE products indicated the existence of two different cDNAs (ara1 [previously named arb1], acc. No. KJ584693 and arab2 [previously named arb2], acc. No. KJ584694), each coding for a different Ar subtype. The open-reading frames (ORFs) were PCR amplified using the Advantage HF kit (Clontech, Westburg, Netherlands) with primer pairs 1512–1514 and 1513–1515 (see Table 2) and random primed-salmon testis cDNA as template. These PCR products were cloned into pcDNA3.1/V5-His TOPO (Life Technologies, Bleiswijk,

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<th>Target</th>
<th>Accession number</th>
<th>Primer name</th>
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<th>Slope</th>
<th>R²</th>
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<td>AY722411</td>
<td>Fw</td>
<td>CACCTCACCTCTGCGACGCTTACAA</td>
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<td></td>
<td>Rv</td>
<td>EAACATTGGAATCTCCATTTCAGTTTAC</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Probe</td>
<td>TTTGCGCTTCTGTTGCCCTGCTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>igf3</td>
<td>JN635414</td>
<td>Fw</td>
<td>GACGGAGGCAAAGATGCAA</td>
<td>2.111^b</td>
<td>0.9929</td>
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<td>Probe</td>
<td>AACTATAGCCATCTGAGTCCCTCGG</td>
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</table>

^a Slope for primer/probe combinations obtained in validation experiments using four 10-fold dilutions of testis cDNA.
^b Slope for primer/probe combinations obtained in validation experiments using five 4-fold dilutions of testis cDNA.
values were calculated with non-linear regression

values, a dose–response
values from IC50
values were calculated using the formula:

\[ \text{IC50 ± SEM} \]

from four treatment groups: control (C), T at 75
and included in these primers, is indicated in italics; R = A or G.

Plasma androgen levels of 11-Ketotestosterone (A) and testosterone (B) in male Atlantic salmon 7 and 14 days after androgen injection. Bars show the mean
Fig. 2.

structs, of which the inserts were sequence verified.

ara1
poly-L-lysin hydrobromide (Sigma–Aldrich, Zwijndrecht, Netherlands) to generate
the transfected cells were transferred to 24-well plates coated with
(mouse mammary tumor virus)-luciferase plasmid. One day later,
expression vector construct in combination with 10
or the
ara2
expression vector construct were incubated at room temperature for
90 min with radioactive tracer ([\(^3\)H]-testosterone), alone or in
combination with unlabeled steroids. Cells were then quickly
washed two times with ice-cold phosphate-buffered saline to
remove unbound tracer, and cell-associated radioactivity was
counted. IC\(_{50}\) values were calculated with non-linear regression
using the GraphPad Prism\textsuperscript{\textregistered} 5 (Version 5.01, GraphPad Software, Inc., USA). To calculate \(K_i\) values from IC\(_{50}\) values, a dose–response
curve of non-labeled testosterone was included in each experi-
ment, and \(K_i\) values were calculated using the formula: \(K_i = (IC_{50} - \text{testosterone})/K_d[\text{[H]}]-\text{testosterone}\).

2.7. Statistical analysis

Data were pooled according to the different treatments, and are
presented as mean ± SEM. To achieve homogeneity of variance,
data were log10 transformed. Differences (significance level of
\(P < 0.05\)) between multiple treatment groups were analyzed by
one-way ANOVA followed by Student–Newman–Keuls test (SNK).
A two-tailed unpaired Student’s t-test was used to compare data
from the same treatment group but sampled 1 or 2 weeks,
respectively, after the start of treatment.

3. Results and discussion

3.1. Plasma steroids levels

Injection of OA and T elevated the plasma levels of 11-KT and T
(Fig. 2). The 11-KT levels were somewhat higher than those found
in the pilot study and in mature Atlantic salmon postsmolts (Melo
et al., 2014) but similar to levels found in spawning anadromous
males (Freeman et al., 1983); elevated 11-KT levels following treat-
ment with OA confirm previous observations that OA is efficiently
converted to 11-KT in vivo in different fish species (e.g. Borg et al.,
1998; Shao et al., 2012). That plasma T levels of 2–3 ng/ml were
found 7 and 14 days after injection of OA alone may reflect the
cross-reaction rate of 5% of our T-antiserum with 11-KT (Schulz,
1985). The T plasma levels found after treatment with T alone
(−20 ng/ml) were comparable to those previously reported in
mature anadromous males (Idler et al., 1971). Administering a 3-
fold higher dose of T (75 instead of 25 \(\mu\)g/g) compared to the pilot
study did not result in higher plasma levels but prolonged the
period of significantly elevated plasma T levels from 4 days in the first
trial (Fig. 1A) to 7 days in the present experiment (Fig. 2A, T-treat-
group). However, 14 days after injection of T, the T plasma

Table 2

<table>
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<tr>
<th>Primer name(^a)</th>
<th>Sequence 5'→3'</th>
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<tr>
<td>1432 (Fw1; 3'-RACE)</td>
<td>CCTCGGACCA/GCTTTACTGAAATGACCA/G</td>
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<tr>
<td>1433 (Fw2; 3'-RACE)</td>
<td>GACCTTGCATCAGGCGTTCGAGC</td>
</tr>
<tr>
<td>1434 (Rv1; 5'-RACE)</td>
<td>AGATATACAGTTGGTCATGTCG</td>
</tr>
<tr>
<td>1435 (Rv2; 5'-RACE)</td>
<td>TCCGAAACTCTCTAGTGCAGTCG</td>
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<tr>
<td>1512 (Fw; PCR)(^b)</td>
<td>TACGCAACATGAGATCCCGTGGTAAAGCCG</td>
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<td>1513 (Fw; PCR)(^b)</td>
<td>TACGCAACATGAGATCCCGTGGTAAAGCCG</td>
</tr>
<tr>
<td>1514 (Rv; PCR)</td>
<td>TTGCTGCTGCTGCTGATTGGTAAAGCCG</td>
</tr>
<tr>
<td>1515 (Rv; PCR)</td>
<td>TTGCTGCTGCTGCTGATTGGTAAAGCCG</td>
</tr>
</tbody>
</table>

\(^a\) Behind the primer name, it is indicated if a primer has a forward (Fw) or reverse
(Rv) orientation, and for which application (3'-RACE, 5'-RACE or PCR) the primer was
used.

\(^b\) The Kozak consensus sequence, preceding the ATG translation initiation codon
and included in these primers, is indicated in italics; R = A or G.

Fig. 2. Plasma androgen levels of 11-Ketotestosterone (A) and testosterone (B) in male Atlantic salmon 7 and 14 days after androgen injection. Bars show the mean
values ± SEM from four treatment groups: control (C), T at 75 \(\mu\)g/g body weight (T75), OA at 25 \(\mu\)g/g body weight (OA25), and the combination of the two androgens
(T75 + OA25). Different lower case letters denote significant differences between treatments within each sampling date. (\(P < 0.05\); one-way ANOVA followed by SNK test).
Asterisks denote means that are significantly different time-wise (\(P < 0.05\); two-tailed unpaired Student’s t-test). The number of males per group is indicated between
brackets under the respective bars.
levels were no longer clearly elevated, so that plasma T levels, next to not reaching the height of 11-KT levels, also decreased faster than 11-KT levels (Fig. 2B). This indicates that T is subjected to more efficient clearance mechanisms than 11-KT. Part of this mechanism possibly is related to the conjugation of steroids to glucuronic acid that has been described in red blood cells in rainbow trout (Schulz, 1986); T but not 11-KT is accepted as substrate by this enzyme activity that prepares T for excretion, thereby reducing its biological half-life time. Still, treatment with T did exert biological effects in our experiments, considering the changes in pituitary and testicular transcript levels (see below). More clearly elevated plasma levels of T may have been present before day 7, as was observed when blood was collected 4 days after steroid treatment in the pilot study (Fig. 1A).

Unexpectedly, after co-injecting OA and T, while still resulting in clearly elevated 11-KT levels, these levels were ~30% lower than after treatment with OA alone (Fig. 2A). Also with respect to T levels, co-injection with OA prevented plasma T levels to reach or maintain the levels found on day 7 in the group treated with T only. Also during natural maturation cycles, plasma levels of 11-KT in the Atlantic salmon were 5– to 6-fold higher than T levels (Freeman et al., 1983). Androgen treatment experiments in stickleback (Gasterosteus aculeatus; Shao et al., 2012) suggested that homeostatic mechanisms are active that prevent androgen plasma levels to reach too high levels, which might compromise other physiological processes, e.g. via immune suppressive effects (Dijkstra et al., 2007). The results of the present experiments point in a similar direction: the increasing doses of OA administered in the pilot study did not lead to increasing plasma levels of 11-KT but prolonged the period during which elevated 11-KT plasma levels were found (Fig. 1B). It appears that homeostatic mechanisms can sense exceedingly high androgen concentrations and respond appropriately, e.g. by up-regulating androgen clearance. Co-treatment with a second androgen may have activated such mechanisms, potentially resulting in ~30% lower 11-KT levels. The stronger effect on T clearance after co-treatment with OA may indicate that this hypothetical mechanism would not only act on 11-KT but also on T, which would then be subjected to two clearance pathways. Clearly, these homeostatic mechanisms deserve further attention in the future.

3.2. GSI, progress of spermatogenesis, and testicular gene expression

The GSI values in the control group did not change during the experimental period (Fig. 3A). Also the high proportion of spermatagonia type Aund in these tests (Fig. 3B, see inset for an example of the morphology of type Aund spermatagonia) are in line with the prepubertal condition of these males. Hence, as expected, the control group males had remained in an immature condition at the end of the experiment: GSI and 11-KT plasma levels as well as the number of type Aund and type Adiff spermatagonia were very similar to immature males kept previously under similar photoperiod and temperature conditions (Fjelldal et al., 2011; Melo et al., 2014).

Seven days after androgen treatment, the GSI levels did not differ significantly among treatment groups (Fig. 3A). After 14 days the group that received the combined treatment (T75 + OA25) showed slightly higher GSI values. Comparing the two consecutive samplings within a treatment group showed that animals that received OA alone displayed higher GSI values in the second week after treatment. We did not count the number of germ cells in cysts containing type Adiff spermatagonia, but it is known that with each cell cycle, the cell number doubles. Hence, the somewhat higher GSI levels in the groups showing consistently elevated androgen levels (i.e. the groups receiving OA25 or T75 + OA25) may reflect the presence of cysts with type Adiff spermatagonia that have gone through one additional cell cycle.

Histological studies were carried out only on the samples collected 2 weeks after androgen injection (Fig. 3B and C); the samples collected for morphological analysis after 7 days were lost. However, since the GSI values did not change after 7 days (see above) and since histological analyses in Atlantic salmon with similar low GSI values showed that the testses contained mainly Aund spermatagonia (Melo et al., 2014), we would not expect major histological changes to have taken place after 7 days. Analysis of the samples collected after 14 days demonstrated that fish showing consistently elevated plasma 11-KT levels presented a more than
5-fold higher number of type Aund spermatogonia than control fish (Fig. 3C). Treatment with T alone had a less prominent effect not reaching statistical significance but it cannot be excluded that a longer period of elevated plasma T levels would have induced a clearer response. The stimulatory effect observed in groups with consistently elevated 11-KT plasma levels most likely reflects a direct androgen effect on the testis, since androgen treatment of gonad-intact, immature salmonid males did not increase but rather decrease the plasma levels of Fsh (Dickey and Swanson, 1998), the only gonadotropin available in immature males at that stage of puberty (e.g. Gomez et al., 1999; Campbell et al., 2003). After all, 11-KT directly stimulated spermatogenesis in testis tissue culture experiments (eel: Miura et al., 1991; zebrafish: Leal et al., 2009). Our morphometric data in conjunction with the only minor changes in GSI levels moreover indicate that while consistently elevated androgen levels induced differentiation of existing type Aund into Adiff spermatogonia, the (self-renewal) proliferation of Aund was not stimulated, because the reservoir of Aund became depleted. Apparently, other or additional signals are required to stimulate the self-renewal proliferation of spermatogonia type Aund, e.g. thyroid hormone, as has been shown recently in zebrafish (Morais et al., 2013).

Elevating experimentally circulating T or 11-KT levels in rainbow trout resulted in partially similar and partially distinct effects on testicular gene expression (Rolland et al., 2013); in juvenile catfish, T inhibited 11-KT-stimulated spermatogenesis (Cavaco et al., 2001). Typically Ar expression and functioning takes place in somatic cells of the testis in vertebrates (Smith and Walker, 2014). We did not study the cellular localization of ara1/2 gene expression in the salmon testis, since the high sequence identity did not allow designing paralogue-specific probes for in situ hybridization purposes. However, in adult zebrafish, the (single) ar mRNA was detected in Sertoli cells contacting type A spermatogonia (de Waal et al., 2008), and Ar-mediated signaling is considered to affect germ cells indirectly, such as via Ar-modulated Sertoli cell gene expression. Examples in fish are the androgen-mediated down-regulation of anti-Müllerian hormone, that inhibited spermatogenesis in juvenile eel (Miura et al., 2002) and adult zebrafish (Skaar et al., 2011), or the androgen-mediated up-regulation of activin, a stimulator of spermatogenesis (Miura et al., 1995).

After 7 days, both androgens clearly down-regulated amh and up-regulated igf3 mRNA levels with no significant difference between the androgen treatment groups (Fig. 4A and B). After 14 days, when plasma T levels were lower compared to the samples collected after 7 days in fish injected with T only, the igf3 mRNA levels had fallen back to the levels of the control group (Fig. 4B), while amh transcript levels remained partially suppressed (Fig. 4A). Remarkably, in this group receiving only T, a more statistically not significant changes in the numbers of spermatogonia type Aund and type Adiff were recorded (Fig. 3B and C). These observations allow two conclusions: (i) the amh transcript levels required more time to clamp back to control levels than igf3 transcript levels required to fall down to control levels; (ii) taking into account that after 14 days, significant changes in the number of spermatogonia type A were only found in fish showing a strong down-regulation of testicular amh and at the same time an up-regulation of igf3 mRNA levels, 7 days of reduced amh and elevated igf3 transcript levels seems insufficient to trigger differentiation of spermatogonia type Aund; triggering and/or sustaining this differentiation may depend on more sustained changes of the transcript levels of these growth factors. Down-regulation of testicular amh mRNA levels following androgen or Fsh treatments (Miura et al., 2002; Skaar et al., 2011; Rolland et al., 2013; Sambroni et al., 2013b; Mazón et al., 2014), or during natural maturation (Maugars and Schmitz, 2008) has been reported previously. However, the present study is the first to show that reduced amh mRNA levels (such as after 14 days of injection with T alone) were not sufficient to allow differentiation of spermatogonia, and that also igf3 mRNA levels needed to be up-regulated for salmon type A spermatogonia to differentiate. Studies in rainbow trout testis provided somewhat incongruous results about the effects of (steroidogenic) gonadotropins and sex steroids on growth factor expression. While Fsh and Lh increased igf3 expression and androgen production, adding androgens to primary testis tissue cultures decreased igf3 transcript levels (Sambroni et al., 2013a,b). Future work will have to resolve the background for these differences; yet additional growth factors and/or binding proteins modulating the biological activities of growth factors or sex steroids may play a role in this regard.

3.3. Pituitary gene expression

3.3.1. lhb and gnrhr

Aromatizable androgens and estrogens directly increased lhb mRNA levels in different fish species (Aroua et al., 2012; Hellqvist et al., 2008; Cheng et al., 2007) and independent lines of evidence demonstrated that this involved an estrogen receptor dependent mechanism (e.g. Wang et al., 2009; Antonompoou et al., 2009; Rebers et al., 2000; Le Dréan et al., 1996). The marked changes we observed in lhb mRNA levels 7 and 14 days after injection with T (alone or in combination with OA), without significant differences over time (Fig. 5A), are well in line with these previous
the more subtle increases in lhb mRNA levels in the groups treated with OA alone (Fig. 5A).

Interestingly, the patterns of pituitary lhb and gnrhr4 mRNA levels (Fig. 5A and B) were quite similar for fish treated with T. Alike findings were described for the black porgy, Acanthopagrus schlegeli, where in vivo and in vitro approaches showed that T (and estradiol) increased pituitary gnrhr mRNA levels (Lin et al., 2010). In fish, two different pituitary cell types produce Lh and Fsh (Nozaki et al., 1990; Kanda et al., 2011), and GnRH triggered the release of both gonadotropins (Levavi-Sivan et al., 2010). While no information is available at present on the pituitary cell type-specific expression of gnrhr genes, we speculate that T, next to increasing lhb mRNA levels, also prepared the Lh-producing pituitary cell type for GnRH-mediated Lh release during the final stages of maturation. Since Lh plasma levels are measurable only during these final stages of maturation in salmonids (Campbell et al., 2003; Gomez et al., 1999; Prat et al., 1996), and since Lh is not detectable in plasma 10, 8, or 5 months before spawning in male salmon, even after steroid treatment (Dickey and Swanson, 1998), we assume that circulating Lh did not play a role in the observed effects of steroid injections on spermatogonial differentiation. Additional support for this conclusion has been provided very recently by genetic evidence, since zebrafish males go through puberty and reproduce despite loss-of-function mutations of Lh or its receptor (Chu et al., 2014).

In salmonids, the start of spermatogenesis is accompanied by increased pituitary fshb mRNA and serum 11-KT levels (Melo et al., 2014; Maugars and Schmitz, 2008; Campbell et al., 2003; Gomez et al., 1999). The steroidogenic activity of Fsh in salmonid males (Planas et al., 1993; Planas and Swanson, 1995) suggests that androgens may serve as feedback signals for pituitary Fsh production and/or release. Our androgen treatments did not alter pituitary fshb mRNA levels significantly (Fig. 5C). Likewise, exogenous T had no effect on pituitary fshb mRNA or Fsh protein levels in gonad-intact male coho salmon 10, 8, and 5 months before spawning (Dickey and Swanson, 1998). As regards Fsh release, however, but also with respect to Fsh synthesis in castrated males, studies in Atlantic and Pacific salmon provided evidence for a negative feedback of sex steroids on Fsh release. Castration during the testis growth phase markedly increased pituitary and plasma Fsh levels, which was reversed by treatment with T or OA (Antonopoulou et al., 1999; Larsen and Swanson, 1997), providing clear evidence for a negative steroid feedback. The absence of steroid effects on the Fsh amount in gonad-intact males may reflect the presence of additional factors of testicular origin also involved in regulating Fsh production and/or release.

In the present study, we generated 11-KT plasma levels as high as in mature, anadromous males, which induced differentiation, but also depleted the population of type Aund spermatogonia in the testis (Fig. 3B and C). Next to a potential role of thyroid hormones in this regard (Morais et al., 2013), it is possible that during natural maturation, when 11-KT levels at the beginning of pubertal testis growth are elevated to 10–20 ng/ml, i.e. 5- to 10-fold above the levels found in immature males (Melo et al., 2014) but still not close to the 80–100 ng/ml in fully mature males (Freeman et al., 1983), pituitary Fsh release may not be (strongly) suppressed, and Fsh might still be able to contribute to stimulate the production of both gonadotropins (Levavi-Sivan et al., 2010). While castration male Atlantic salmon parr were treated with T or T in combination with an aromatase inhibitor (ATD), pituitary Lh levels that were very high after administering T only, were reduced after adding ATD, but were still higher than in castrated control fish (Antonopoulou et al., 1999). This may suggest that there was also a weak, Ar-mediated contribution to the overall stimulation of lhb gene expression in the present study, which may explain
3.4. Androgen receptors

Two different ar cDNAs (ara1 and ara2), each encoding a different Ar, were isolated from Atlantic salmon testis. We choose to follow the nomenclature as suggested by Douard et al. (2008). Phylogenetic analysis of their deduced amino acid sequences (Fig. S1) revealed that both Atlantic salmon receptors (Ara1 and Ara2) belong to the Ara branch (Douard et al., 2008) of androgen receptors, while additional cloning attempts showed no evidence for the existence of Atlantic salmon Ar forms belonging to the Arb branch. Fig. S1 also shows that both Atlantic salmon Ars belong to the same clade as the two rainbow trout Ar forms, with Atlantic salmon Ara1 displaying the highest homology with rainbow trout Ara1, and Atlantic salmon Ara2 with rainbow trout Ara2. As also rainbow trout misses Arb forms, salmonids may have lost arb genes, while one additional genome duplication in salmonids resulted in two paralogous ara genes, ara1 and ara2 (Douard et al., 2008).

Using HEK293T cells, transfected with either expression vectors for ligand binding studies (Fig. S2) revealed that Ara1 has a higher affinity for both, T (Kd of 1.6 ± 0.1 nM) and 11-KT (Kd of 1.7 ± 0.1 nM) than Ara2 (T Kd of 9.2 ± 0.6, 11-KT Kd of 4.5 ± 0.1 nM). Using [3H]-testosterone and different androgens as non-radioactive competitors, we found that the synthetic androgen 17α-methyltestosterone (MT) and the natural androgens 5α-dihydrotestosterone (DHT), T and 11-KT behaved as high affinity ligands for Ara1, with Ki concentrations of 0.3–2 nM. Other androgens such as precursors and intermediate products of 11-KT biosynthesis like androstenedione (A), 11β-hydroxyandrostenedione (OHA), 11β-hydroxytestosterone (OHT) and adrenosterone (OA)
showed an intermediate affinity (50 nM–0.5 μM; Fig. 6A). A similar pattern was found when studying Ara2 (Fig. 6B), except that for all androgens tested, ~10-fold higher steroid concentrations were required to achieve the same displacement (Fig. 6C).

Using co-transfections of either the ara1 or the ara2 expression constructs, in combination with an AR-sensitive reporter gene (MMTV) to examine the activity as ligand-activated transcription factor in general, confirmed the results of the ligand binding studies (Fig. 7): focusing on high affinity androgens, we found that ~14-fold lower concentrations of MT were sufficient for a half-maximal activation of reporter gene expression comparing Ara1 and Ara2. While T and 11-KT showed EC50 concentrations of 2 and 6 nM, respectively, for Ara1 (Fig. 7A), these steroids were unable to achieve half-maximal activation of reporter gene expression via Ara2 even at 1 μM concentrations (Fig. 7B).

Rolland et al. (2013) showed that T and 11-KT had similar but also distinct effects on testicular gene expression in rainbow trout. We speculated that the two Ara paralogous genes present in salmon (Doudart et al., 2008) might have distinct characteristics as regards androgen binding, activity as transcriptional regulator, and/or expression patterns. Preliminary data on the testicular transcript levels of the two Atlantic salmon paralogues do not suggest large differences in expression (ratio of Cq-values in 10 immature males for the two paralogues was 0.98 ± 0.02). Moreover, both Ara forms failed to clearly differentiate between T and 11-KT in binding assays and showed only slight differences in activation assays. Accordingly, both T and 11-KT had similar effects on testicular Igf3 and amh transcript levels on day 7. Nevertheless, even in vertebrates with only a single androgen receptor gene, androgens can have different effects in different cell types. In mammals, for example, these cell type-specific effects are considered to reflect the cell type-specific expression of proteins interacting with the androgen receptor (Johnson and O’Malley, 2012; Watson et al., 2012).

4. Conclusion

Elevating plasma androgen levels directly stimulated spermatogonial differentiation in immature Atlantic salmon testis tissue. Regulatory mechanisms may operate in vivo that prevent circulating androgen levels from reaching very high plasma levels, in particular as regards T. Only consistently elevated androgen levels can induce consistent changes in testicular amh and igf3 transcript levels, associated with an increase in the number of differentiated spermatogonia that took place at the expense of undifferentiated spermatogonia. Depletion of the latter may be based, at least in part, on missing Fsh stimulation. The Atlantic salmon androgen receptors do not differentiate between T and 11-KT.

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Appendix A. Supplementary data

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References


