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ASPECTOS MORFOFISIOLÓGICOS DA MATURAÇÃO SEXUAL DO SALMÃO DO ATLÂNTICO (*SALMO SALAR*)

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ASPECTOS MORFOFISIOLÓGICOS DA MATURAÇÃO SEXUAL DO SALMÃO DO ATLÂNTICO (*SALMO SALAR*)

Tese apresentada ao Curso de Pós-Graduação em Biologia Celular da Universidade Federal de Minas Gerais, como requisito parcial para obtenção do título de doutor em Biologia Celular.

Área de **concentração**: Biologia Celular

Orientador: Prof. Dr. Luiz Renato de França

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Programa de Pós-Graduação em Biologia Celular

ATA DA DEFESA DE TESE DE DOUTORADO DE

MICHELLE CAROLINA DE MELO

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Às **treze horas** do dia **16 de janeiro de 2015**, reuniu-se, no Instituto de Ciências Biológicas da UFMG, a Comissão Examinadora da Tese, indicada pelo Colegiado de Programa, para julgar, em exame final, o trabalho final intitulado: **"ASPECTOS MORFOFISIOLÓGICOS DA MATURAÇÃO SEXUAL DO SALMÃO DO ATLÂNTICO (SALMO SALAR)"**, requisito final para obtenção do grau de Doutora em Biologia Celular, área de concentração: **Biologia Celular.** Abrindo a sessão, o Presidente da Comissão, **Ph.D. Luiz Renato de França**, após dar a conhecer aos presentes o teor das Normas Regulamentares do Trabalho Final, passou a palavra à candidata, para apresentação de seu trabalho. Seguiu-se a arguição pelos examinadores, com a respectiva defesa da candidata. Logo após, a Comissão se reuniu, sem a presença da candidata e do público, para julgamento e expedição de resultado final. Foram atribuídas as seguintes indicações:

Prof./Pesq.	Instituição	Indicação
Ph.D. Luiz Renato de França	UFMG	APROVADA
Dr. José Carlos Nogueira	FASEH	APROVADA
Dr. Hugo Pereira Godinho	PUC/MG	APROVADA
Dr. Gilmar Bastos Santos	PUC/MG	APROVADA
Dr. José Dias Corrêa Júnior	UFMG	APROVADA

Pelas indicações, a candidata foi considerada: APROVADA

O resultado final foi comunicado publicamente à candidata pelo Presidente da Comissão. Nada mais havendo a tratar, o Presidente encerrou a reunião e lavrou a presente ATA, que será assinada por todos os membros participantes da Comissão Examinadora. **Belo Horizonte, 16 de janeiro de 2015.**

Ph.D. Luiz Renato de França (Orientador) LUIZ RENATO DE FRANÇA

Dr. José Carlos Nogueira

Dr. Hugo Pereira Godinho

Dr. Gilmar Bastos Santos

Dr. José Dias Corrêa Júnior

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Coordenadora do Programa de Pós Graduação
em Biologia Celular ICB / UFMG

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AGRADECIMENTOS

Embora uma tese seja, pela sua finalidade acadêmica, um trabalho individual, há contribuições de natureza diversa que não podem e nem devem deixar de ser realçados. Por essa razão, desejo expressar os meus sinceros agradecimentos:

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Adiff = espermatogônias diferenciadas

Amh = hormônio anti-Mülleriano

Aund = espermatogônias indiferenciada

Fsh = hormônio folículo estimulante

GnRH = hormônio liberador de gonadotrofinas

GnRHr = receptor para ,o hormônio liberador de gonadotrofinas

HPG = hipotálamo-pituitária-gônada

Igf3 = fator de crescimento semelhante à insulina tipo-3

Lh = hormônio luteinizante

Lhr = receptor para Lh

SSC = espermatogônia-tronco

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Resumo

O ciclo de vida dos salmonídeos é complexo e composto de variadas estratégias de vida e esta complexidade se reflete na vida reprodutiva destes peixes. Na aquicultura, a puberdade precoce representa ainda o principal problema na criação comercial do salmão do Atlântico, em decorrência dos consideráveis efeitos negativos desta condição no crescimento e bem-estar animal. O presente estudo teve como objetivos fornecer evidências morfofisiológicas acerca dos efeitos de fatores exógenos (fotoperíodo e salinidade) e endogénos (tratamento com esteróides sexuais) sobre os eixos neuroendócrinos que controlam o crescimento e a maturação sexual do salmão do Atlântico. Utilizando o modelo de maturação em salmão *postsmolt* observamos que diferentes fatores ambinetais modularam diferentes aspectos da espermatogênese, o que resultou numa ativação diferencial da hipófise e testículo, sendo a salinidade um fator estimulatório no início da puberdade e o fotoperíodo na fase final da espermatogênese. Estudando a participação dos esteróides sexuais na fase inicial da maturação sexual concluimos que elevados e constantes níveis plasmáticos de andrógenos estimularam diretamente a diferenciação espermatogonial em detrimento das espermatogônias indiferenciadas e que mecanismos regulatórios podem operar in *vivo* para impeder que os níveis de andrógenos circulantes mantenham-se elevados, em particular no que diz respeito à testosterona. O número aumentado de espermatogônias diferenciadas apresentou-se associado às alterações consistentes nos níveis de RNA mensageiro testicular para o hormônio anti-Mülleriano (*amh)* e fator de crescimento semelhante à insulina tipo-3 (*igf3).*

Abstract

The life cycle of salmon is complex and endowed with different life strategies and this complexity is applied to the reproductive life of these fish. In aquaculture, early puberty is still the main problem in commercial farming of Atlantic salmon due to the considerable negative effects of this condition on growth and welfare. The present study aimed to provide morphophysiological evidences regarding the effects of exogenous factors (photoperiod and salinity) and endogenous (treatment with sex steroids) on the neuroendocrine axes that control the growth and sexual maturation of Atlantic salmon. Using the model of maturation in *postsmolt* salmon we observed that different environmental factors modulated different aspects of spermatogenesis, resulting in a differential activation of the pituitary gland and testis; with salinity stimulating the onset and the shorter photoperiod the completion of spermatogenesis. Studying the effects of sex steroids in early sexual maturation we concluded that high and constant plasma levels of androgens directly stimulated spermatogonial differentiation at the expense of undifferentiated spermatogonia and that regulatory mechanisms can operate in vivo to avoid that circulating androgen levels are maintained high, in particular regarding to testosterone. Also, the increased number of differentiated spermatogonia was associated with consistent changes in testicular transcript levels for anti-Müllerian hormone *(amh)* and Insulin-like growth factor-3 (*igf3).*

1. INTRODUÇÃO E REVISÃO DE LITERATURA

1.1 Peixes Teleósteos

Os peixes constituem cerca de 50% das quase 50 mil espécies de vertebrados e exibem grande diversidade em sua biologia, morfologia e em habitats ocupados (Nelson, 1994). O sucesso dos teleósteos é atribuído a uma série de adaptações que aperfeiçoaram sua respiração, locomoção, nutrição e principalmente a reprodução (Moyle & Cech, 1996), caracterizada por uma das mais variadas estratégias reprodutivas (Nagahama, 1983; Redding & Patiño, 1993; Pudney 1996; Le Gac & Loir, 1999; Nakatani et al., 2001). Nesse contexto, a liberação dos gametas para fertilização externa; o desenvolvimento de órgãos especializados para fertilização interna; os variados tipos de comportamento e de cuidado com a prole; e a migração reprodutiva, seja nas formas de "piracema" ou naquelas acompanhadas por grandes alterações osmóticas, são exemplos da complexidade reprodutiva dos teleósteos (Le Gac & Loir, 1999). Devido ao grande potencial econômico representado pela piscicultura no mercado mundial de alimentos, torna-se crucial conhecer mais detalhadamente as particularidades relacionadas à biologia reprodutiva destes vertebrados.

1.2 Espermatogênese em teleósteos

A espermatogênese é um processo cíclico, complexo, altamente conservado e bastante coordenado que ocorre no interior de estruturas denominadas espermatocistos, ou cistos, formados quando uma única espermatogônia-tronco (espermatogônia do tipo A) é completamente envolvida pelos prolongamentos das células de Sertoli (Grier, 1993; Pudney, 1993; 1995; Schulz et al., 2010). Uma vez formado o cisto, é considerado que a espermatogônia do tipo A pode sofrer dois diferentes tipos de divisão: i) divisão para auto-renovação, garantindo assim fertilidade a longo prazo; e ii) divisão para prover duas células filhas, as quais se desenvolvem, após um número de divisões mitóticas específico para cada espécie, em espermatócitos que completarão a

meiose para produzir células haplóides denominadas de espermatozóides (Schulz et al., 2010). O balanço entre estes dois tipos de divisões é fortemente coordenado. Assim, quando o mesmo é alterado rumo à auto-renovação, a formação de seminomas pode ser desencadeada. Por outro lado, se este balanço segue em direção à diferenciação, a população de espermatogônias-tronco pode ser depletada e a espermatogênese interrompida (De Rooij & Grootegoed, 1998).

Baseado em características morfofuncionais, a espermatogênese pode ser dividida em três fases: (a) fase proliferativa ou espermatogonial, caracterizada pela auto-renovação das espermatogônias tronco e/ou diferenciação das mesmas rumo a (b) fase meiótica ou espermatocitária, na qual o material genético é duplicado, recombinado e segregado; e (c) fase de diferenciação ou espermiogênica, em que as espermátides passam por transformações morfogenéticas que culminam na formação dos espermatozóides, tornando-se os mesmos aptos para fertilizar o ovócito após sua espermiação e maturação (Russell et al., 1990; Sharpe, 1994; Eddy, 1999; Schulz & Miura, 2002).

Durante a fase mitótica, o número de gerações espermatogoniais pode variar entre as diferentes espécies de peixes (Ando et al., 2000). Pelo fato da nomenclatura das diferentes gerações de espermatogônias ser bastante variada em peixes, recentemente, tentativa foi feita no sentido de se classificar as mesmas usando terminologia empregada para vertebrados superiores (Schulz et al., 2010). Baseado em critérios morfológicos, as células espermatogoniais podem ser classificadas em dois tipos: tipo A e tipo B. Funcionalmente, as espermatogônias do tipo A podem ser subdivididas em espermatogônias indiferenciadas (Aund), incluindo-se as espermatogônias-tronco (SSC); e espermatogônias diferenciadas (Adiff). As espermatogônias Aund originam as espermatogônias Adiff, que compartilham algumas características morfológicas com as Aund. O comprometimento irreversível com a diferenciação/maturação e alterações morfofuncionais, como por exemplo, maior quantidade de heterocromatina, resulta na

formação de espermatogônias do tipo B que usualmente passam por várias e rápidas divisões, propiciando grande aumento no número deste tipo celular. Neste sentido, o melhor critério para discriminar espermatogônias do tipo B inicial (B inicial) do tipo B tardia (B tardia) baseia-se no tamanho celular/nuclear e no número de células por cisto (Schulz et al., 2010).

Uma vez que as espermatogônias do tipo B originam os espermatócitos primários, estes irão progredir para a divisão meiótica. A prófase da primeira divisão meiótica é um processo longo subdividido em cinco fases com características morfofuncionais específicas, denominadas de pré-leptóteno, leptóteno, zigóteno, paquíteno e diplóteno. Este último tipo de espermatócito finalizará a primeira divisão meiótica dando origem aos espermatócitos secundários, os quais, por sua vez, passam rapidamente pela segunda divisão meiótica que irá resultar na formação de células haplóides, as espermátides (Billard, 1984; Billard, 1986; Russell et al., 1990).

A fase espermiogênica é caracterizada por drástica transformação das espermátides provenientes da divisão meiótica seguida por sua liberação no lume dos túbulos seminíferos, processo este conhecido como espermiação (Hess & França, 2007). Esta transformação engloba fenômenos marcantes como a perda substancial de citoplasma, cujo excesso é fagocitado pelas células de Sertoli em forma de corpos residuais, e a compactação do DNA, resultando em notável redução do volume celular (Billard, 1986). Ainda durante a fase espermiogênica ocorre formação da peça intermediária e flagelo, originando os espermatozóides, células estas aptas a fertilizarem os ovócitos (Russell et al., 1990; Stoumboudi & Abraham, 1996).

1.3 Controle endócrino da maturação testicular

À semelhança de outros vertebrados, o início da maturação sexual em peixes é regulado pelo eixo hipotálamo-pituitária-gônada (HPG). Fatores exógenos ambientais, como fotoperíodo, temperatura, propriedades químicas da água ou oferta de alimentos, e endógenos, como sinais

internos relacionados ao crescimento/adiposidade, ou feedback de esteróides sexuais provenientes dos testículos, são de alguma forma percebidos e transduzidos pelo cérebro em sinais neuroendócrinos (Taranger et al., 2009, Zohar et al., 2010; Levavi-Sivan et al., 2010; Schulz et al., 2010). O principal neurohormônio envolvido nesse processo de transdução de sinais é o hormônio liberador de gonadotrofinas (GnRH), o qual está presente em diversas regiões cerebrais e hipotalâmicas nos peixes e cujos receptores específicos (GnRHr) estão presentes nas células gonadotróficas da adenohipófise regulando, assim, a secreção pela pituitária das gonadotrofinas hormônio folículo estimulante (Fsh) e hormônio luteinizante (Lh) (Redding & Patiño, 1993; Bosma et al., 1996; Kah et al., 2007).

Ambas as gonadotrofinas, Fsh e Lh, são composta por uma subunidade comum α - e uma subunidade específica β para cada hormônio, e desempenham papel fundamental na fisiologia testicular, garantindo a produção de hormônios/fatores de crescimento envolvidos na maturação de células germinativas. Por sua vez, estes hormônios/fatores integrado com fatores exógenos e endógenos exercem efeitos de feedback no eixo cérebro-pituitária controlando a síntese e secreção de Fsh e Lh.

Apesar de grande similaridade entre os vertebrados, peixes teleósteos apresentam algumas particularidades em relação aos hormônios gonadotróficos e as suas atividades biológicas. No testículo, o receptor do Lh (Lhr) é expresso nas células de Leydig presentes no compartimento intertubular e sua principal função é estimular a produção de esteróides sexuais. Já o receptor de Fsh (Fshr), diferentemente dos mamíferos, é expresso nas células de Sertoli e de Leydig. Portanto, além de atuar sobre as células de Sertoli, estimulando a produção de diversos e importantes fatores de crescimento para as células germinativas, o Fsh também estimula a esteroidogênese (Ohta et al., 2007; García-López et al., 2009).

1.4 Salmão do atlântico (*Salmo salar*)

O salmão do atlântico é um peixe teleósteo pertencente à Família Salmonidae, da ordem dos Salmoniformes. É uma espécie migradora anádroma, ou seja, vive no mar até atingir o estado adulto e sobe os rios para se reproduzir. O ciclo de vida dos salmonídeos é complexo e dotado de variadas estratégias de vida (Thorpe,1994; Fleming, 1996, Figura 1).

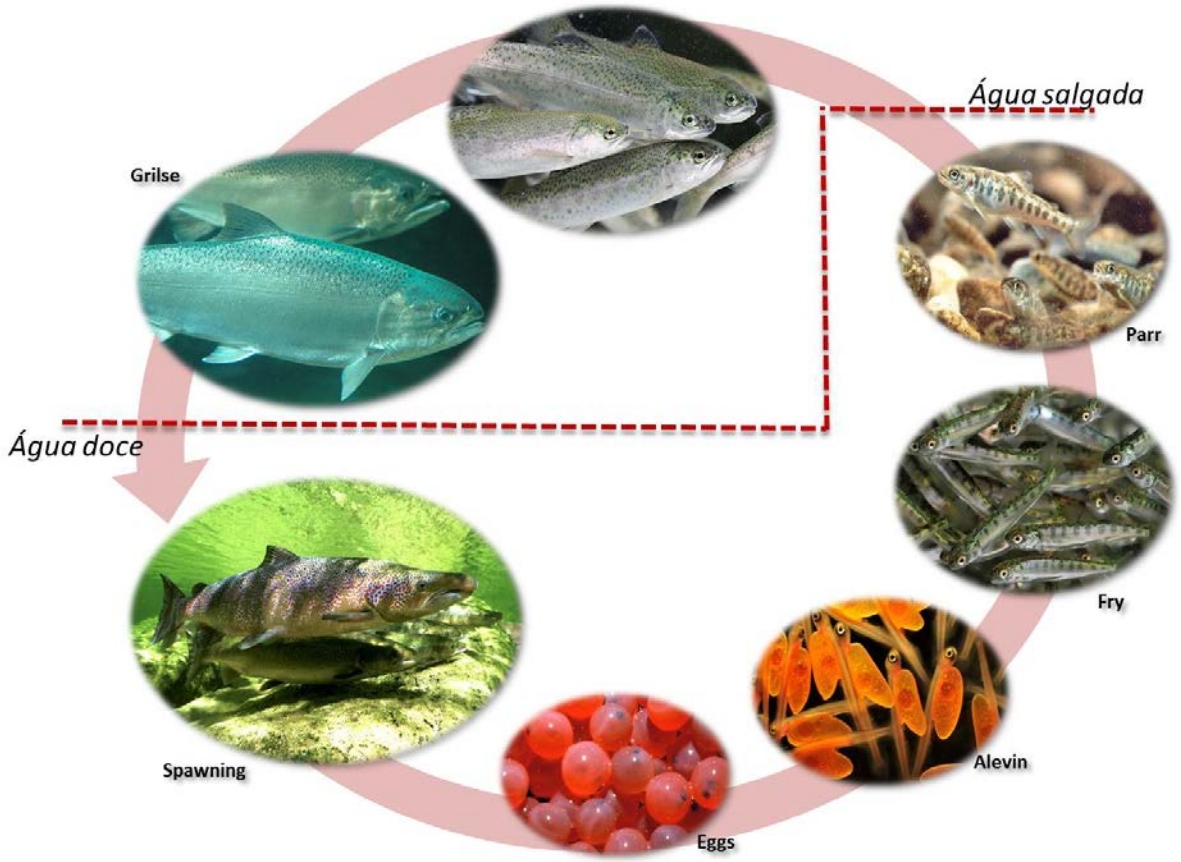

Figura 1 – Ciclo de vida do salmão do atlântico

O salmão do atlântico se reproduz no outono em água doce (Outubro-Novembro) e os ovos fertilizados desenvolvem durante o inverno e eclodem (*alevin*) entre Abril e Maio e medem cerca de 2 cm de comprimento. Durante quatro a seis semanas, os alevinos alimentam-se das suas próprias reservas. Após este período, começam a alimentar-se de larvas de insetos (*fry*) e podem permanecer por um ou vários anos na água doce até se tornarem *parr*. No final da

primavera/início do verão, o *parr* imaturo pode se tornar fisiologicamente adaptado ao ambiente salino (*postsmolt*) e migra para o mar. Após 1 a 4 anos vivendo no mar, o salmão do Atlântico torna-se sexualmente maduro (*grilse*) retornando para seus locais de reprodução nos rios. A maioria dos adultos morre de exaustão quando termina a época de reprodução e apenas uma minoria volta ao mar, podendo reproduzir-se uma segunda vez (Fleming, 1996). No estágio *parr* o salmão pode atingir a maturidade sexual nos rios após um ano de vida e, apesar do tamanho bem menor, competir com o (*grilse*) para fecundação dos ovos nos locais das desovas. Estima-se que 40% dos ovos liberados são fertilizados pelo salmão do atlântico no estágio *parr* (Fleming, 1996). No ano seguinte, machos *parr* podem tornar-se sexualmente maduros novamente ou sofrer a esmoltificação e migrar para o mar.

1.5 Problemas associados à puberdade precoce e condições ambientais que promovem ou inibem a maturação sexual do salmão do Atlântico.

A puberdade precoce representa ainda o principal problema na criação comercial do salmão do Atlântico devido aos consideráveis efeitos negativos desta condição no crescimento somático, utilização deste teleósteo como alimento, bem como na qualidade de sua carne e saúde e bem-estar animal (Taranger et al., 2010). Todos os processos fisiológicos envolvidos na reprodução de peixes são controlados por inúmeros fatores endócrinos ao longo do eixo HPG e estes processos são modulados diretamente por fatores ambientais, que podem direta ou indiretamente determinar o sucesso reprodutivo de uma espécie (Ribeiro & Moreira, 2012). Por exemplo, nas espécies de regiões temperadas que desovam na primavera ou início do verão, o crescimento gonadal é estimulado por fotoperíodo longo, geralmente em combinação com altas temperatura, como no caso de carpas. Já nas espécies que desovam no outono ou início do

inverno, o crescimento gonadal é estimulado por diminuição do fotoperíodo, como ocorre na maioria dos salmonídeos (Baldisserotto, 2002).

Pelo fato de alterar a taxa de maturação sexual entre os salmões, na aquicultura o uso de regimes de fotoperíodo ou iluminação artificial e a manipulação da temperatura representam interessantes abordagens. Assim, Fjelldal et al. (2011) mostraram que a elevação da temperatura da água (16ºC) e luz contínua induziram a maturação sexual em cerca de 90% dos salmões investigados no estágio *postsmolt*, poucos meses após a transferência dos mesmos para a água do mar. Entretanto, o uso comercial destas abordagens pode ser bastante comprometido por resultados imprevisíveis dos tratamentos entre os diferentes locais e anos. Em particular, parece que uma elevada incidência da maturação sexual ocorre após verões intensos, apesar do uso de fotoperíodo inibitório (Bromage et al., 2001; Taranger et al., 2010). Assim, proporção variável de peixes tornam-se sexualmente maduros antes de serem capturados para consumo, permanecendo em ambiente salino durante todo o seu ciclo de maturação. Esta permanência resulta em desidratação e alta mortalidade dos peixes, representando um dos principais problemas no cultivo de salmão do Atlântico (Taranger,1993). Pois, sob a influência de hormônios esteróides sexuais após início da puberdade, o sistema osmorregulatório é reajustado novamente para que o salmão migre de ambiente hiperosmótico (marítimo) para ambiente hiposmótico (água doce) durante a piracema (Taranger,1993). Este problema é mais pronunciado em machos, uma vez que eles alcançam a puberdade/maturação sexual em idade e tamanho inferior aos das fêmeas e este evento pode ocorrer naturalmente em distintos períodos ao longo de sua vida, tais como *parr* e *grilse*.

1.6 Justificativa

Embora diversos estudos tenham demostrado que em salmonídeos as respostas fisiológicas são moduladas por variações nos fatores ambientais (Taranger et al., 2010), o conhecimento acerca das consequências da manipulação destes fatores sobre os eixos neuroendócrinos que controlam o crescimento e a maturação sexual do salmão do Atlântico é ainda pouco conhecido. O modelo de maturação em ambiente salino utilizado por Fjelldal et al. (2011), no qual os salmões exibem peso corporal ainda relativamente pequeno (± 200g), apresenta várias vantagens do ponto de vista experimental, uma vez que a maturação sexual ocorre rapidamente, permitindo, em poucos meses, o estudo deste processo desde o início da puberdade até a reprodução. Além disso, o menor peso corporal à puberdade permite a realização de experimentos onde a temperatura, o fotoperíodo e a salinidade podem ser total e facilmente controlados, possibilitando utilizar número razoavelmente maior de peixes, bem como maior número de repetições. Desta forma, este novo modelo de maturação permite realizar estudo temporal da maturação *postsmolt*, com ênfase na evolução do processo espermatogênico e seu controle endócrino, o que é crucial para a compreensão do início da puberdade "fora de época", que usualmente ocorre em salmões criados comercialmente.

1.7 Objetivos

A utilização do modelo de maturação em salmão *postsmolt* associado com ferramentas moleculares e abordagens morfofuncionais foi realizada na Holanda e Noruega e possibilitou melhor entendimento acerca da espermatogênese em salmão do Atlântico. Neste contexto, o capítulo 2 **(Salinity and photoperiod modulate pubertal development in Atlantic salmon (*Salmo salar*)** do presente estudo teve como principal objetivo obter conhecimento mais

detalhado da espermatogênese de peixes expostos a diferentes condições ambientais, i.e fotoperíodo e salinidade, e teve os seguintes importantes aspectos abordados:

- Análise, na adenohipófise, da expressão de genes relacionados ao início da puberdade/maturação sexual (*gnrhr4, fshβ e lhβ*) na presença de diferentes condições de salinidade e fotoperíodo;
- Investigação do efeito das diferentes condições experimentais no desenvolvimento do processo espermatogênico, buscando-se correlacionar este evento com possíveis alterações na expressão de genes da pituitária;
- Avaliação dos possíveis efeitos das diferentes condições ambientais na esteroidogênese em salmão *postsmolt*.

Já no capítulo 3 **(Androgens directly stimulate spermatogonial differentiation in juvenile Atlantic salmon (*Salmo salar*)**, teve como objetivo avaliar o papel dos diferentes esteróides sexuais (testosterona e 11-cetotestosterona) nos processos fisiológicos envolvidos no início da maturação sexual do salmão do Atlântico, e os seguintes importantes aspectos foram estudados:

- Mensuração dos níveis séricos de hormônios sexuais após administração de solução contendo diferentes esteróides;
- Análise, no testículo, da expressão de genes relacionados ao início da puberdade (*amh* e *igf3*), procurando-se correlacionar a expressão dos genes estudados com o progresso da espermatogênese;
- Avaliação, na adenohipófise, do efeito dos diferentes esteróides sexuais na expressão de genes envolvidos na maturação sexual (*gnrhr4, fshβ e lhβ*);
- Avaliação da atividade biológica dos diferentes esteróides sexuais, através dos receptores de andrógenos.

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ARTIGO 1

Salinity and photoperiod modulate pubertal development in Atlantic salmon (*Salmo salar*)

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Abstract

The Atlantic salmon shows substantial life cycle plasticity, which also applies to the timing of puberty. While it is characterized by the activation of the brain–pituitary–gonad axis, many morphophysiological aspects of puberty and the influence of environmental conditions, such as water salinity, are not well understood in fish. Here, 12-month-old Atlantic salmon coming from an out-of-season smoltification regime in December were exposed to freshwater (FW) or seawater (SW) at 16 °C to stimulate puberty under a 24-h constant light (LL) or 12 h light:12 h darkness (LD) photoperiod. These four treatment groups (FWLL, SWLL, FWLD, and SWLD) were studied from January to March. Next to 11-ketotestosterone (11-KT) plasma levels, the expression of pituitary genes (*gnrhr4*, *fshb*, and *lhb*) and spermatogenesis was quantified. When spermatogonial proliferation started, *fshb* mRNA levels increased steeply and began to decrease when spermatogonial mitosis approached completion and most germ cells had reached meiotic or post-meiotic stages. Conversely, *lhb* mRNA levels increased progressively during spermatogenesis. Most males in all treatment groups matured, but exposure to SW resulted in the strongest stimulation of the onset of spermatogenesis and elevation of pituitary *gnrhr4* and *fshb* mRNA levels. Later on, the LD photoperiod accelerated, irrespective of the salinity, the completion of spermatogenesis, associated with higher *lhb* mRNA and 11-KT plasma levels than in the LL groups. We find that both salinity and photoperiod modulated different aspects of spermatogenesis, and resulted in a differential activation of pituitary and testis functions; SW stimulating the onset and the shorter photoperiod the completion of spermatogenesis.

Key Words
- Atlantic salmon
- salinity
- photoperiod
- pituitary gene expression
- spermatogenesis

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Introduction

The Atlantic salmon (*Salmo salar*) is a cold-water-adapted, anadromous teleost that experiences seasonal changes in temperature and photoperiod in fresh and seawater (SW) and exhibits a substantial plasticity in the timing and routing of its life cycle (Taylor 1991, Hutchings & Jones 1998). This plasticity also applies to puberty that can occur at different stages of the life cycle (Taranger *et al.* 2010). While it is known that environmental conditions like photoperiod and water temperature can affect the timing of puberty, the specific roles of these

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conditions and their possible interactions are not well established (Fjelldal *et al.* 2011). Moreover, sexual maturation occurs at different water salinities in salmon, such as at full salinity in the ocean (grilse and multi sea-winter salmon), as precocious parr in freshwater (FW; Jonsson & Jonsson 2007), and also in fjords or estuarine areas with brackish water (Jonsson *et al.* 2001). However, information on the possible effects of salinity on puberty is missing.

In salmon aquaculture, early puberty is a common problem compromising somatic growth, harvest quality, and fish welfare (Bromage *et al.* 2001, Taranger *et al.* 2010). Despite these negative impacts, in particular, affecting males that normally reach puberty at a lower age and smaller size than females, there is yet limited knowledge on both physiological mechanisms triggering puberty in salmon or other fishes and modulatory effects of environmental factors on the functioning of these mechanisms (Taranger *et al.* 2010). Recently, Fjelldal *et al.* (2011) found that high (16 °C) water temperature in combination with continuous light induced puberty at the postsmolt stage just after SW transfer. This induced postsmolt maturation provides an excellent experimental model to study physiological processes involved in puberty and to study how these processes are modulated by different environmental factors.

We used this model to the examine effects of salinity and photoperiod on entry and completion of puberty in males. Prior to commencing the experimental treatments at the postsmolt stage, an out-of-season photoperiod regime was applied in FW from October to December to induce smoltification (Stefansson *et al.* 1991). Samples were collected every 3 weeks during the next 3 months (January to March) to analyze the expression of key genes in the pituitary, plasma androgen levels and to evaluate spermatogenesis quantitatively. This unique data set allowed new conclusions as regards physiological processes involved in the onset as well as completion of spermatogenesis and modulation of these processes by water salinity and photoperiod.

Materials and methods

Experimental design

The experiments took place at Matre Research facility (61°N), which is part of the Institute of Marine Research, Bergen (Norway). On September 29, 1600 Atlantic salmon presmolts were distributed over 16 experimental tanks of 500 l with 100 fish per tank (body weight 151±19 g (mean±S.D.). The fish were kept under constant light (LL) in FW until October 20, when an out-of-season smoltification regime (Berge *et al.* 1995, Arnesen *et al.* 2003) was initiated: 6 weeks of a short-day photoperiod LD (0900–2100 h; 12:12) was followed by 4 weeks of LL from December 1. Water temperature was maintained at 11.8 ±0.7 °C until January 4. On January 5, after completion of smoltification, eight tanks were moved back to LD 12:12 while the remaining eight tanks were kept on LL. The body weight was 376±155 g at this time. The tanks were supplied with either FW or SW 35ppt (SW), and either LL or LD, creating four experimental groups (FWLL, FWLD, SWLL, and SWLD; Fig. 1) with four replicate tanks per group. To stimulate maturation (Fjelldal *et al.* 2011), the water temperature was increased in all tanks on

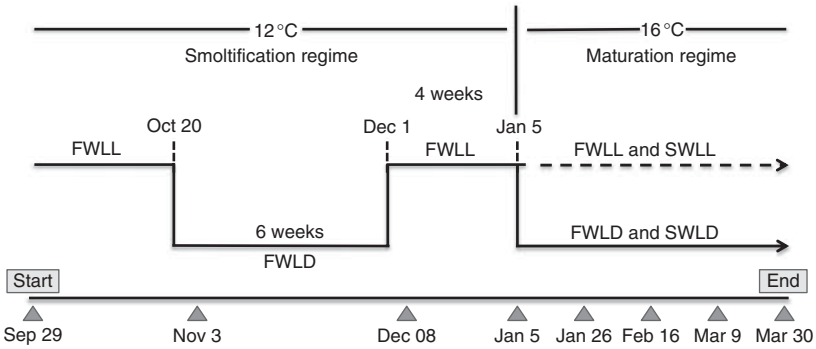

Figure 1
Experimental design to investigate the effects of different environmental conditions on sexual maturation of Atlantic salmon postsmolts. All fish underwent a smoltification regime at 12 °C from September 29 to January 5. Fish were then split into four experimental groups with four replicate tanks, each receiving a particular maturation regime from January 5 to March 30 by elevating water temperature to 16 °C. The fish in the four different groups were exposed to the following environmental conditions: FWLL, SWLL, FWLD and SWLD, where FW, fresh water; SW, salt water; LL, continuous light and LD, 12 h light:12 h darkness. Samples were collected on the dates indicated by arrowheads.

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January 12 and was maintained at 15.5±1.1 °C until completion of the experiment on March 30. Fish were sampled every 3 weeks both during the smoltification regime and after establishing the four experimental groups for collection of tissue and blood samples (Fig. 1).

Sampling

Fish were killed in water containing 10 mg/l of the anesthetic metomidate (Syndel, Victoria, BC, Canada). Body weight was recorded, blood was collected from the caudal veins, and gonads were excised. Testes were weighed and fixed in Bouin for 24 h and stored in 70% ethanol. Blood was centrifuged and plasma was stored at −80 °C. The gonadosomatic index (GSI) was calculated as GSI (%)=gonad weight (g)×100/total body weight (g). The pituitary was excised, snap–frozen in liquid nitrogen, and then stored at −80 °C until RNA extraction.

Measurement of plasma concentration of 11-ketotestosterone

Prior to RIA, individual plasma samples were mixed with Milli-Q water (containing 0.05% (w/v) NaN_3) in the ratio 1:2 and incubated at 80 °C for 1 h. After centrifugation (at 21 000 ***g*** for 30 min), the supernatant was stored at −20 °C until RIA. Quantification of 11-ketotestosterone (11-KT) plasma levels was carried out using a specific RIA (Schulz 1985).

Testis histology and morphometry

For routine histological analysis, sections of 5 μm were stained with periodic acid–Schiff and hematoxylin/eosin. The progress through spermatogenesis was analyzed quantitatively using the software ImageJ and ten non-overlapping digital images per fish were randomly taken (Olympus-AX-70; Nikon Digital Camera DXM 1200) at 600× magnification. 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 in the different phases of spermatogenesis. We distinguished undifferentiated type A spermatogonia ($SPGA_{und}$; single cells with no or very little heterochromatin in the nucleus, one or two prominent nucleoli, and a nuclear diameter of ∼15 μm), differentiated type A spermatogonia ($SPGA_{diff}$; two or more cells with some heterochromatin and a nuclear diameter of ∼10 μm), and type B spermatogonia (SPGB; groups of cells with a high amount of heterochromatin and a nuclear diameter below 8 μm). Counts for primary and secondary spermatocytes were combined. Different stages of spermiogenesis were counted collectively as spermatids. Spermatozoa in the lumen of the seminiferous tubules were counted when the intersection fell on the sperm head.

Next to the quantitative evaluation, we noted for each male the furthest developed germ cell type, being either $SPGA_{und}$, $SPGA_{diff}$, SPGB, spermatocytes and/or spermatids (SC-ST), or spermatozoa (SZ). Finally, when free spermatozoa filled the tubular lumen, the males were qualified as spermiated (SP). The typical histological appearance of these stages is presented in Supplementary Figure 1, see section on supplementary data given at the end of this article.

Immunohistochemistry

Proliferation of germ ($SPGA_{und}$ and $SPGA_{diff}$) and Sertoli cells was assessed by immunocytochemical localization of the proliferation marker phosphorylated histone H3 (pH3; Hendzel *et al.* 1997, Cobb *et al.* 1999). Three sections of 5 μm that were at least three sections apart from each other were used for detection of pH3 as described by Almeida *et al.* (2008), except that the primary antibody was detected by undiluted HRP-conjugated goat anti-rabbit IgG (Brightvision Immunologic, Duiven, The Netherlands) for 30 min. To quantify proliferation, 25 non-overlapping fields were randomly chosen and analyzed using a Nikon Digital Camera DXM 1200 connected to Olympus-AX-70 at 1000× magnification. The number of pH3-positive cells was counted in the 25 fields and expressed as the average of cells per field.

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

Frozen salmon pituitaries were homogenized in 1 ml tubes containing Trizol reagent from the IPrepTM Trizol PLUS RNA Kit (Invitrogen) and zirconium oxide beads in a Precellys 24 Homogenizer (Bertin, Villeurbanne, France), followed by RNA isolation. Next, cDNA synthesis was performed with 2 μg of each total RNA sample using a Superscript VILO cDNA synthesis kit (Invitrogen). To estimate the relative salmon *gnrhr4* (see Table 1), *lhb*, and *fshb* (Andersson *et al.* 2013) mRNA levels, TaqMan assays were performed, as described previously (de Waal *et al.* 2008). The levels of elongation factor *ef1a* mRNA (Olsvik *et al.* 2005) served as endogenous control RNA, which remained stably expressed under the different experimental conditions.

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Table 1 Primers and TaqMan fluorogenic probe used in real-time PCR assays to determine the relative *gnrhr4* mRNA levels in Atlantic salmon pituitaries

Target	Genbank accession number	Primer name	Sequence 5′–3′	PCR efficiency (%)
gnrhr4	KF225730	Fw	TCAACCCACTGGCGATCAAT	101.8
		Rv	CGTGATGGTCACACTGTGGAATA	
		Pr	AGTGTGATTCTGTCTGTTCCCCAGATGCTG	

All TaqMan assays were performed in 10 µl reaction volumes and their Cq values were determined in a 7900HT Real-Time PCR System (Applied Biosystems) using default settings. Relative mRNA levels, calibrated to the first sampling, were calculated as reported previously (Bogerd *et al*. 2001, Good *et al*. 2012).

Statistical analysis

Data were analyzed using STATISTICA v11 (StatSoft, Inc. 2012; Tulsa, OK, USA). To achieve homogeneity of variance, data were log10 transformed; GSI data were arcsin transformed. The number of germ cells was analyzed by one-way ANOVA followed by Tukey's multiple comparisons test, or two-tailed unpaired Student's *t*-test when necessary, with a significance level of $P<0.05$. As regards GSI, 11-KT levels, and gene expression data presented according to the sampling date, the time-wise effects within the treatment groups were also tested with one-way ANOVA followed by Tukey's multiple comparisons test, using all males until January 5, and using the maturing males within each treatment group from January 26. In addition, all males from January 5 were compared with the maturing males in the four treatment groups at January 26 with one-way ANOVA followed by Dunnet's *post hoc* test, using the January 5 sample as controls. Subsequently, effects of salinity and photoperiod on maturing males from January 26 were analyzed by two-way ANOVA within each sampling date, and in all males by stage of germ cell development by one-way ANOVA, followed by Newman–Keuls *post hoc* test comparing groups formed according to the most advanced germ cell stage. The GSI values of the eight males sampled on January 5 showed a bimodal distribution (<0.04 vs >0.05; $n=4$ in both modes). After sorting the data on pituitary gene expression, androgen plasma levels, and proliferation activity in an immature and a maturing group, the differences between these groups were analyzed by two-tailed unpaired Student's *t*-test ($P<0.05$). The incidence of maturation among the groups was compared with a χ^2 test using the SWLL group as control and significance level of $P<0.05$. Data on the graphs are presented as mean ± S.E.M.

Results

Morphological analysis of testis maturation

Until January 5, SPGA were the only germ cells present (Fig. 2b), composed predominantly of $SPGA_{und}$ with only a small fraction of $SPGA_{diff}$ (Fig. 3a). Thereafter, SPGB (Fig. 2c) or meiotic and postmeiotic (spermatids and spermatozoa) germ cells (Fig. 2d) were also present in most males. However, between three and 16 males sampled from January 26 to March 30 stayed immature and showed testes with SPGA only (Fig. 2a). The SWLL group showed the highest incidence of maturation (93%), although 70–80% maturation was found in the other groups. Analyzing Sertoli and germ cell proliferation in testes containing only SPGA revealed mainly single cell proliferation (Fig. 2b inset) with no (Sertoli cells) or minor (SPGA) differences between sampling dates (data not shown). In testes recruited into maturation, also smaller and larger germ cell clones were proliferating (Fig. 2c inset), heralded by a shift to the prominent presence of $SPGA_{diff}$ and SPGB in all treatment groups on January 26 (Fig. 3b). Compared with the fish kept in FW, the fish kept in SW (hatched and cross-hatched columns) had significantly more germ cells that had progressed to the stage of SPGB, at the expense of SPGA (Fig. 3b). Comparing the FW groups, the LD photoperiod had a slight stimulatory effect, since we observed less $SPGA_{diff}$ and a tendency to more SPGB than in the FWLL group (Fig. 3b).

From February 16, meiotic (primary and secondary spermatocytes) and postmeiotic (spermatids and spermatozoa) germ cells were found in the maturing testes of all the four groups (Fig. 3c, d, and e). The counts for SPGA became very small and ranged between 0.3 and 1.1 germ cell per field in all groups, with somewhat higher values in the FWLL and lower values in the SWLL and FWLD groups (data not shown). The counts for SPGB and spermatocytes were similar among groups (Fig. 3c), i.e. spermatogonial proliferation and meiosis proceeded in all groups.

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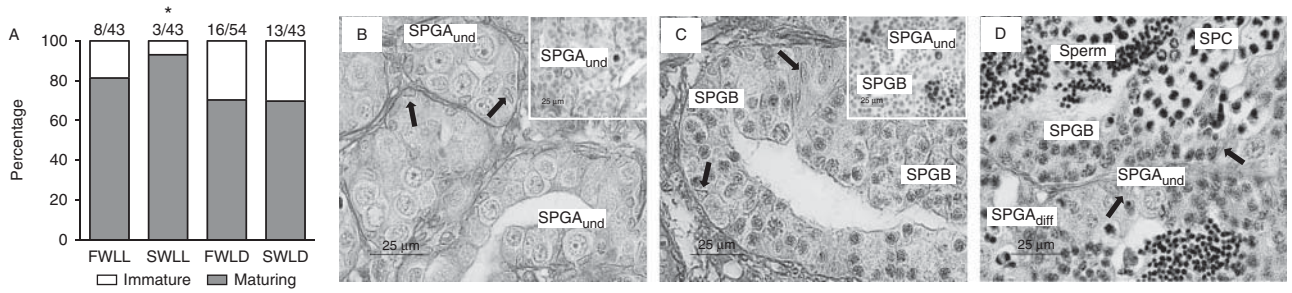

Figure 2
Percentage of males committed to sexual maturation, quantification of cell proliferation in the testis, and testicular histology of Atlantic salmon postsmolts. (a) Percentage of salmon males recruited into sexual maturation. The first number above the bar graph represents the total number of immature fish sampled between January 26 and March 30, the second number represents the total number of fish analyzed. Asterisk indicates the group, which showed a higher percentage of maturing males ($P<0.05$, χ^2-test). (b) Histology of an immature testis sampled on January 5, containing mainly undifferentiated type A spermatogonia (Aund). A low level of mainly single cell proliferation activity is found represented as brown staining in cell nuclei (inset). (c) Salmon testis recruited into sexual maturation, sampled on January 26, showing type B spermatogonia (SPG B) and clearly elevated cell proliferation activity (inset), including single cells, small (top left on inset), and large groups (down right on inset) of germ cells. (d) Testis containing germ cells at different stages of spermatogenesis sampled on February 16. Aund, undifferentiated type A spermatogonia; Adiff, differentiated type A spermatogonia; SPG B, type B spermatogonia; SPC, spermatocyte; FW, fresh water; SW, salt water; LL, continuous light; LD, 12 h light:12 h darkness. Arrows in (b), (c) and (d) indicate Sertoli cell nuclei. A full colour version of this figure is available online at http://dx.doi.org/10.1530/JOE-13-0240.

A different pattern emerged from analyzing the samples collected in March (Fig. 3d and e). While exposure to SW induced earlier formation of SPGB (January 26) and postmeiotic cells (February 16) than in the FW groups, in March, fish under LD showed higher counts for spermatozoa and lower counts for spermatogonia and spermatocytes compared with fish under LL, irrespective of the salinity. On March 30, the number of spermatids had also decreased, indicating that completion of spermiogenesis was approaching. Spermatogenesis still proceeded well in the SWLL and FWLL groups since spermatozoa appeared (FWLL) or increased further (SWLL) on March 9 (Fig. 3d). This development continued toward March 30 (Fig. 3e) but the number of spermatozoa was still lower than in the LD groups.

Overall effects of salinity and photoperiod on recruitment into puberty

The data collected from maturing males were subjected to a two-way ANOVA, testing for effects of salinity, photoperiod, and their interactions within each sampling

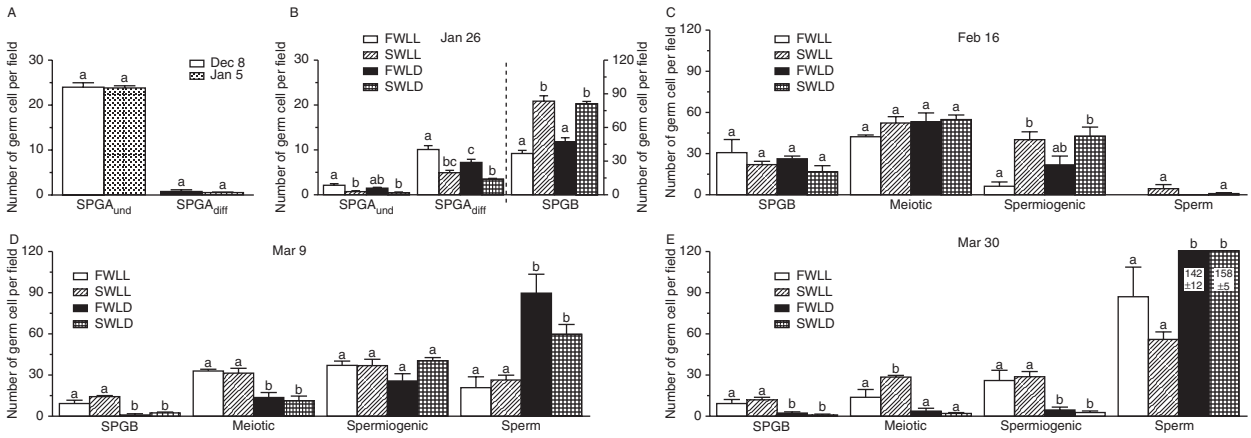

Figure 3
Quantitative evaluation of spermatogenesis. The bar graphs show the mean numbers of different types of germ cells counted per microscopic field at 600× magnification and their S.E.M. from immature fish (a) sampled on December 8 (black bars) and January 5 (grey bars) and at different dates during the maturation regime (b, c, d and e) when different treatment groups were exposed to freshwater (FW) or salt water (SW), constant light (LL) or 12 h light:12 h darkness (LD) resulting in the following four groups: FWLL, SWLL, FWLD and SWLD (legends indicated in the graphs). Bars labeled with different letters showed statistically significant differences in their mean values ($P<0.05$; one-way ANOVA followed by Tukey's test). Aund, undifferentiated type A spermatogonia; Adiff, differentiated type A spermatogonia; SPG B, type B spermatogonia.

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Table 2 Significant ($P<0.05$) differences using two-way ANOVA in GSI, plasma androgen, or pituitary gene expression levels resulting from exposure to different water salinities or photoperiod regimes are indicated by the capital letters S or P respectively according to sampling date. A significant interaction is indicated by the capital letter I

	Jan 26	Feb 16	March 9	March 30
GSI	S, P	P	S, P	S, P
11-KT	P	P	P	P
gnrhr4	S, P	S, P	P	S, P, I
fshb	S, P	P	P, I	S, P
lhb	P	P	P	P

date from January 26 until March 30. A summary of the analysis is given in Table 2 and the detailed results from the two-way ANOVA on effects of salinity and photoperiod within each sampling date are provided in Supplementary Table 1, see section on supplementary data given at the end of this article. The photoperiod regime significantly modulated the maturational response on all sampling dates and parameters studied. In addition, significant but more selective effects of salinity were found, showing that GSI and *gnrhr4* and *fshb* mRNA levels but not plasma androgen and pituitary *lhb* mRNA levels were modulated. Significant interactions between the effects of salinity and photoperiod were noted for *fshb* on March 9 and for *gnrhr4* on March 30.

GSI and 11-KT plasma levels

The average GSI values increased gradually between September 29 and January 5 (Fig. 4a inset). On January 26 and onward, only maturing fish were taken into account for calculating GSI values. The GSI increased significantly in the maturing males in all treatment groups by January 26 (one-way ANOVA followed by Dunnet's *post hoc* test using all males in January 5 as controls). The two groups kept in SW showed 1.6- or 2.1-fold higher values than the respective FW groups (Fig. 4a), indicating that transfer to SW further stimulated the onset of testis growth. The GSI increased five- to tenfold until February 16 in all groups, reaching values between 3.9 and 9.6. While the GSI in fish in SW was no longer different from their FW counterparts, the fish kept under LD showed 1.7- to twofold higher GSI levels than their LL counterparts. On March 9, the GSI values in the two LD groups were slightly (8–7.4%) or clearly (9.6–5.6%) lower compared with 3 weeks earlier, while the GSI reached high values of 7.7 and 9.6% in the two LL groups. Toward March 30, the GSI values decreased clearly in the groups under LD (Fig. 4a) but only slightly (1.1- and 1.2-fold) in the groups under LL. As more germ cells had developed into spermatozoa in the LD groups on March 30 (see Fig. 3e), the more strongly decreasing GSI is in line with the loss of cellular material during spermiogenesis. Maximum GSI values were reached at different sampling dates but showed a similar range (7.6–9.6%) in all treatment groups.

Average 11-KT plasma levels ranged between 1 and 1.3 ng/ml from September to December (Fig. 4b inset). On January 5, a sixfold increase in the mean 11-KT levels was observed. On January 26, 11-KT plasma levels had increased again significantly in maturing males under LD and an alike tendency was found under LL, reaching mean values between 10 and 20 ng/ml (Fig. 4b). A similar pattern was observed on February 16, with no significant changes compared with the previous sampling. However, on March 9 and 30, both LD groups showed 11-KT plasma levels exceeding two- to threefold those of their LL counterparts (Fig. 4b).

Pituitary gene expression

gnrhr4 Pituitary *gnrhr4* mRNA levels increased gradually from September to January 5, reaching statistical significance on January 5 (Fig. 5a), and further increased two- to 4.6-fold by January 26 in all groups. The two SW groups showed 1.5- to 1.8-fold higher expression levels than their FW counterparts. On February 16, the groups kept in SW still showed significantly higher (1.7- and 2.1-fold) *gnrhr4* mRNA levels than the FW groups. A different pattern emerged when analyzing the March samples, where fish under LD showed 1.5- and 2.3-fold higher *gnrhr4* mRNA levels than under LL (March 9), although on March 30, the SWLL group had reached the high level of the LD groups as well.

Gonadotropin β-subunits The first significant increase in *fshb* mRNA levels was recorded on January 5 (Fig. 5b inset). The *fshb* mRNA levels kept increasing strongly (six- to 24-fold) in maturing fish and reached very high levels already on January 26 in all groups (Fig. 5b). High levels (0.7- to 1.4-fold change compared to January 26) were maintained until February 16 with no statistical differences between the groups, before decreases to a third of the previous levels were recorded on March 9 in the FWLD group, and both LD groups that continued to decline toward the final sampling when the levels were

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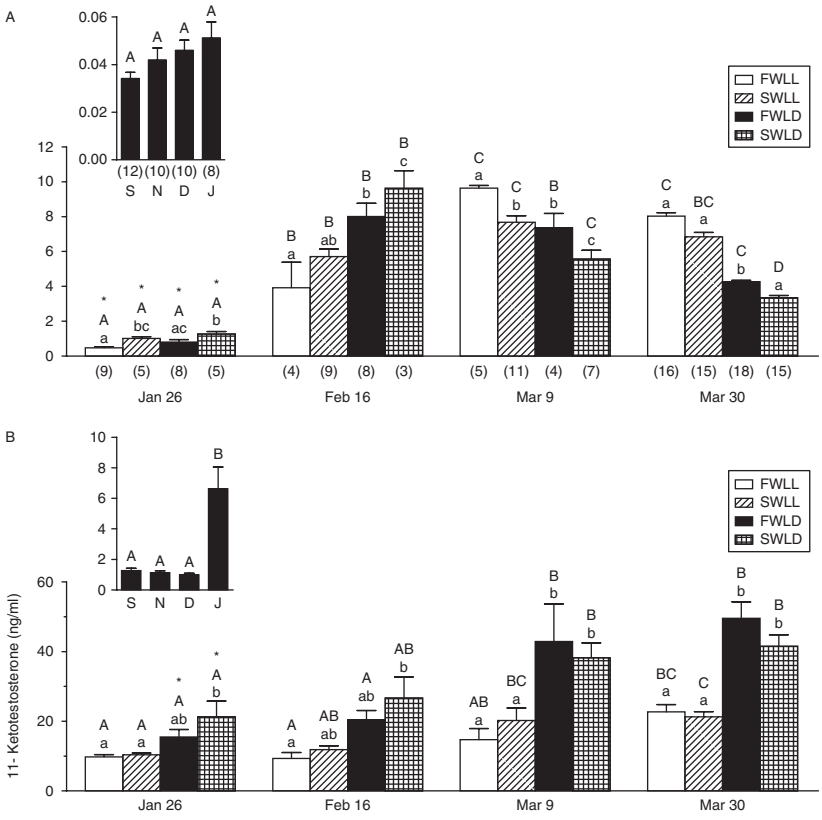

Figure 4

Gonadosomatic index (GSI; a) and 11-ketotestosterone plasma levels (b) of male postsmolt Atlantic salmon during the progression of spermatogenesis. The inset bar graph shows the values of fish sampled between September (S) 29 and January (J) 5. Bars show the mean values and their S.E.M. from the different treatment groups: FWLL, SWLL, FWLD and SWLD (legends are indicated in the graphs). Capital letters in the inserts and in the figures denotes samples that are significantly different time-wise ($P<0.05$; one-way ANOVA followed by Tukey's *post hoc* test). Asterisks above the bars on January 26 denote values that are significantly different from the January 5 sample (one-way ANOVA followed by Dunnet's *post hoc* test using January 5 as controls). Lower case letters denote significant differences between treatments within each sampling date from January 26 onwards ($P<0.05$; two-way ANOVA followed by SNK *post hoc* test). The number of individuals per group is indicated under the respective bars.

again halved (Fig. 5b). The LL groups, on the other hand, had maintained elevated *fshb* mRNA levels on March 9 (0.8- to 1.4-fold change compared to the previous sampling). Thereafter, the level in fish under LL decreased toward March 30 only among those kept in FW. The *fshb* mRNA levels in the SWLD group on January 26 were higher than in all other groups, while on March 30 the lowest levels were found in the FWLD group that ranged significantly below its LL counterpart also on March 9.

Also *lhb* mRNA levels increased significantly for the first time in the pituitaries sampled at the beginning of January. From then on, *lhb* mRNA levels kept increasing in the maturing fish, and elevated levels were maintained until the end of March. A separation of the groups exposed to different photoperiods became evident already on January 26, with higher levels in the LD groups. This difference kept increasing such that in all the following samplings, pituitary *lhb* mRNA levels of the LD groups were twice as high as in the LL groups.

The speed of germ cell development depended on the salinity and photoperiod conditions experienced by the maturing males. To obtain the developmental profiles across treatment groups, we sorted the data of all males according to the most developed type of germ cell present in the testis (Fig. 6). Stage-dependent, significant differences were found for all parameters. In general, low levels were characteristic of fish with testes containing SPGA, and significant increases were associated with the first appearance of SPGB. Except for *fshb* mRNA levels, which already reached a high plateau at this early stage, all other parameters further increased significantly toward meiosis and beyond (Fig. 6). The *fshb* transcript levels also contrasted with the other parameters by starting to decrease with the appearance of spermatozoa. A rather

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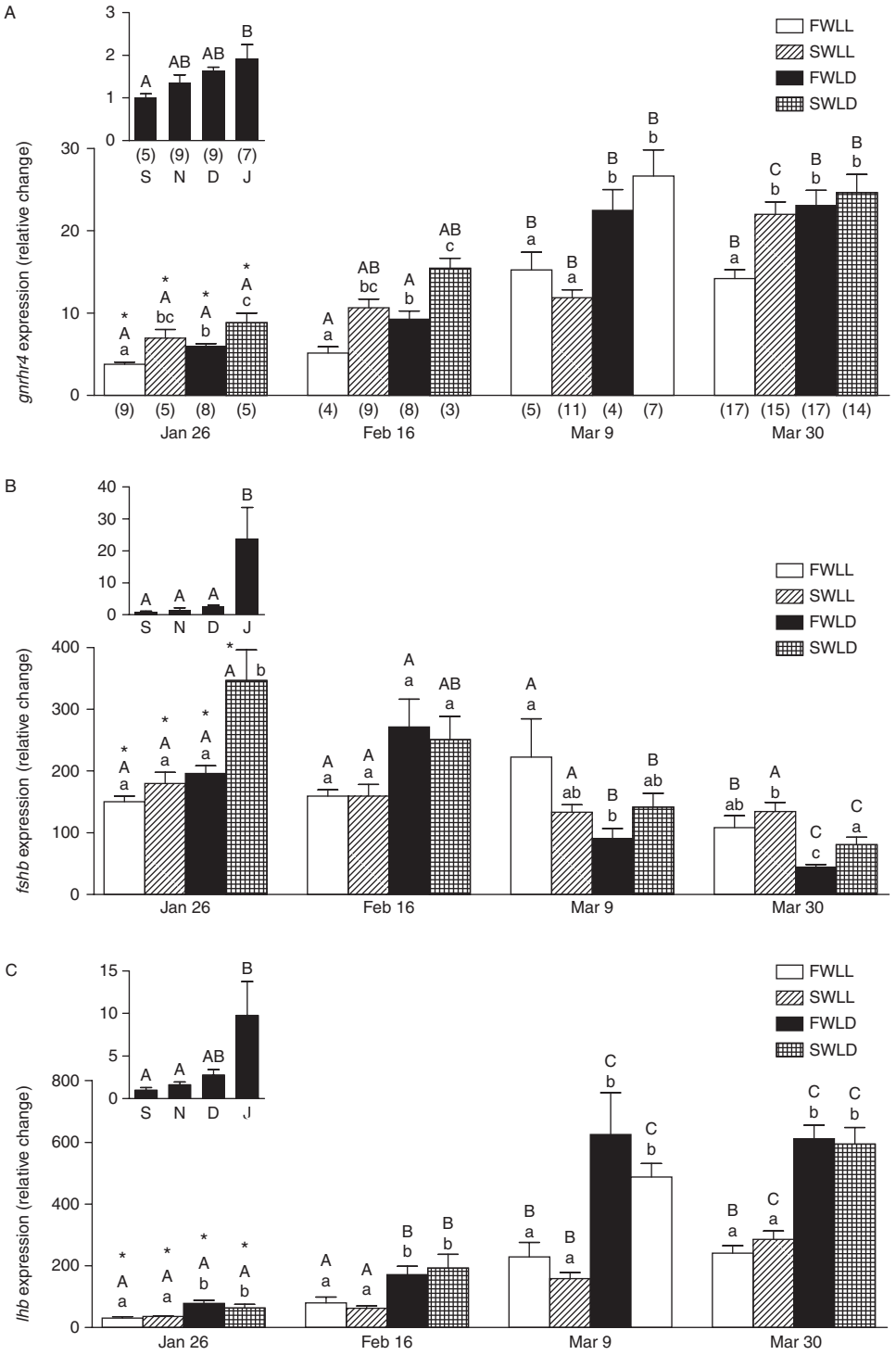

Figure 5

Relative pituitary mRNA levels of the gnrhr4 (a), fshb (b) and lhb (c) genes in Atlantic salmon postsmolts. The inset bar graphs show the pituitary mRNA levels of fish sampled between September (S) 29 and January (J) 5. Bars show the mean values and their S.E.M. from the different treatment groups: FWLL, SWLL, FWLD and SWLD (legends are indicated in the graphs). Capital letters in the insets and in the figures denote samples that are significantly different time-wise ($P<0.05$; one-way ANOVA followed by Tukey's *post hoc* test). Asterisks above the bars on January 26 denote values that are significantly different from the January 5 sample (one-way ANOVA followed by Dunnet's *post hoc* test using January 5 as controls). Lower case letters denote significant differences between treatments within each sampling date from January 26 onwards ($P<0.05$; two-way ANOVA followed by SNK *post hoc* test). The number of individuals per group is indicated under the respective bars.

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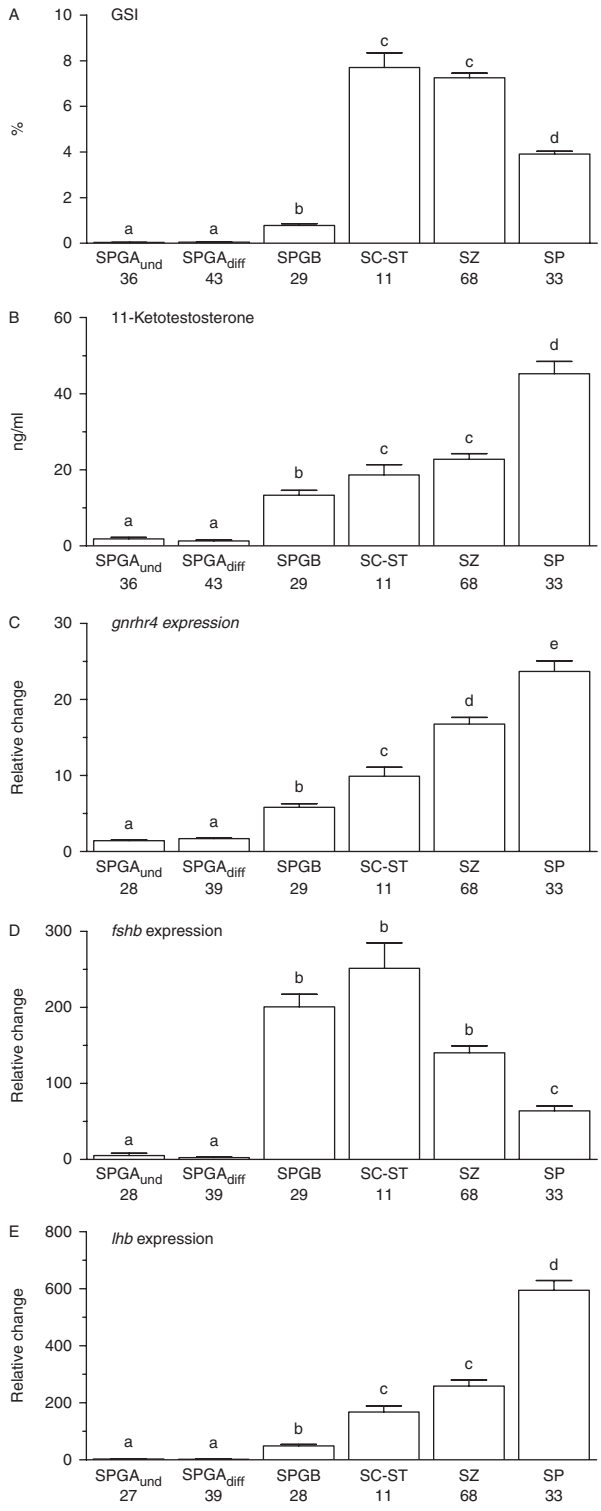
limited variation was typical for this data set, suggesting that the stage of spermatogenesis, but not the treatments, had a strong influence on the changes observed. Hence, salinity and photoperiod conditions affected the timing of development, but not the mass of germ cells formed (GSI), plasma androgen, or pituitary gene expression levels, typically found at specific stages of spermatogenesis.

On January 5, just before the maturation regime with elevated water temperature started, increases in gene expression and plasma androgen levels were recorded, but changes in testicular histology were not observed (all testes contained SPGA; see Fig. 3a). The GSI values of the eight males sampled showed a bimodal distribution (see above Statistical analysis). Sorting proliferation, pituitary gene expression, and plasma androgen levels according to the GSI values (below 0.04%, considered immature; above 0.05%, considered maturing; Fig. 7) revealed statistically significant differences for all parameters. It appears that at the very beginning of puberty, the pituitary gonadotrops became activated in part of the males sampled on January 5 that moreover showed elevated plasma androgen levels and increased testicular cell proliferation activity.



Discussion

There is a substantial plasticity in both age and size at puberty in fish. The timing of recruitment into puberty is sensitive to changes in environmental conditions such as photoperiod, food availability, or temperature. The Atlantic salmon is a prominent example in this regard and can reach sexual maturation as parr, grilse, or multi sea winter salmon (Taylor 1991, Hutchings & Jones 1998, Garcia de Leaniz *et al.* 2007), and recent work has shown that postsmolt maturation can be induced immediately after smoltification (Fjelldal *et al.* 2011). While many studies have investigated physiological mechanisms triggering puberty in fish, original aspects of this study are to study effects of different salinities (in combination with two photoperiod regimes) on the timing of pubertal development, and the detailed morphological analysis of testis development that accompanies analyzing pituitary key gene expression and plasma androgen levels.

Figure 6
Gonadosomatic index (GSI; a), plasma 11-ketotestosterone (11KT; b), relative pituitary mRNA levels of gnrhr4 (c), fshb (d) and lhb (e) in all sampled fish sorted according to the most advanced germ cell stage; Aund, Adiff, SPGB, spermatocytes and/or spermatids (SC–ST), or spermatozoa (SZ). When free, spermiated spermatozoa filled the tubular lumen and represented the by far dominating germ cell type, the males were assigned to the group spermiation (SP). Lower case letters denote significant differences between the stages ($P<0.05$; one-way ANOVA followed by SNK *post hoc* test). The number of individuals per group is indicated under the respective bars.

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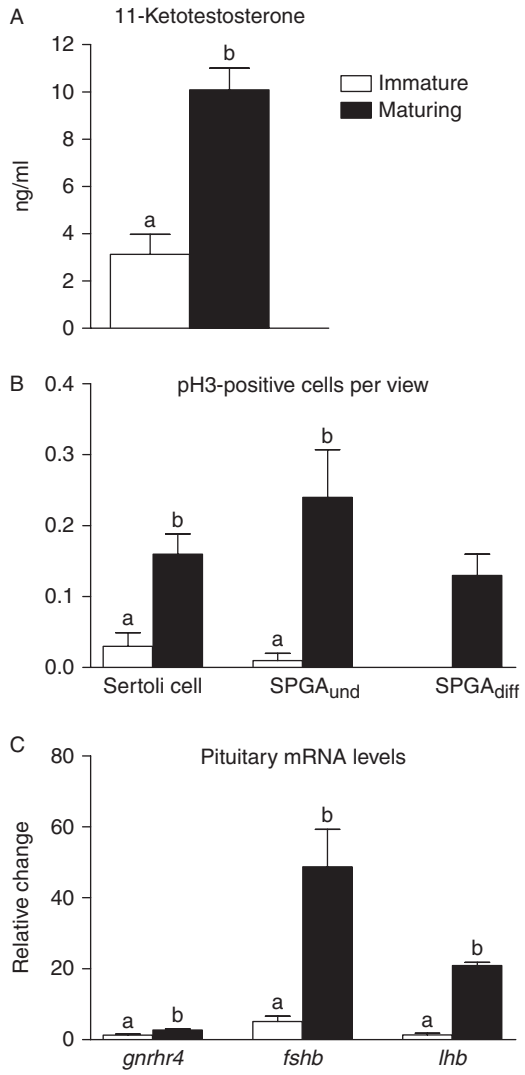

Figure 7
Analyses of different parameters (11-ketostestorone, testicular cell proliferation and pituitary gene expression) in male Atlantic salmon postsmolts. The bar graphs show the mean values and their S.E.M. in immature fishes (open bars) and maturing fishes (black bars) on January 5. Bars labeled with different letters showed statistically significant differences in their mean values ($P<0.05$; two-tailed unpaired Student's *t*-test). Aund, undifferentiated type A spermatogonia; Adiff, differentiated type A spermatogonia.

Our analyses showed that some (mainly single cell) proliferation is found in immature testes from males sampled before or after the start of the maturation regime. We propose that this proliferation activity reflects the allometric growth of the gonads but is not associated with a recruitment into maturation. Accordingly, the GSI in this group of individuals remained low.

Pituitary gene expression, plasma androgen levels, and testicular proliferation activity were clearly elevated by January 5 in half of the males (Fig. 7). This early activation prior to the start of the maturation regime was somewhat surprising but might reflect a stimulatory effect of the continuous light treatment used at the end of the smoltification regime; similar photoperiod conditions stimulated the onset of both parr and postsmolt maturation (Thrush *et al.* 1994, Duncan & Bromage 1998, Fjelldal *et al.* 2011, Skilbrei & Heino 2011). Also the high body weight (c. 200–600 g in this study, compared to normally <100 g) and the late time of the year for the induced, out-of-season smoltification may have allowed maturation to start. Last but not least, the smoltification regime also included a 6-week LD photoperiod (Fig. 1) that may have increased the incidence of maturation. In masu salmon parr, a short photoperiod accelerated maturation, associated with an activation of preoptic GNRH neurons, and an increase in pituitary Fsh levels (Amano *et al.* 1999, 2001). Collectively, these conditions may have activated the brain–pituitary axis at the beginning of January. Planas *et al.* (1993) and Planas & Swanson (1995) showed that Fsh stimulated testicular 11-KT release, probably mediated by the Fsh receptor, which is also expressed by Leydig cells in fish (Ohta *et al.* 2007, García-López *et al.* 2009, 2010, Chauvigné *et al.* 2012). Elevated 11-KT plasma levels were associated with the appearance of SPGB in rainbow trout (*Oncorhynchus mykiss*; Scott & Sumpter 1989) and Chinook salmon (*Oncorhynchus tshawytscha*; Campbell *et al.* 2003). This study is the first to show in an *in vivo* setting in fish that increased proliferation activity of Sertoli cells as well as spermatogonia type A_{und} and A_{diff} are early signs of recruitment into puberty at the beginning of January. Also with respect to *fshb* mRNA levels, Fsh, but not Lh, is elevated in the blood of salmonids at the onset of pubertal development (Swanson *et al.* 1989, Oppen-Berntsen *et al.* 1994, Gomez *et al.* 1999), and *fshb* transcript levels and Fsh plasma levels were quite well correlated during puberty in male rainbow trout (Gomez *et al.* 1999). This opens the possibility that Fsh – next to activating androgen release – has stimulated Sertoli and germ cell (SPGA) proliferation to support recruitment into puberty. Luckenbach *et al.* (2010) showed that pituitary *gnrhr* transcript levels were upregulated in male coho salmon (*Oncorhynchus kisutch*) preparing to enter puberty, an observation in agreement with our finding. Finally, as mentioned earlier, Amano *et al.* (1999, 2001) reported activation of GNRH neurons and increased pituitary Fsh contents in response to environmentally stimulated puberty in masu salmon. Collectively, these data suggest that recruitment into puberty may involve GNRH receptor-mediated activation of Fsh release that

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stimulates androgen production and germ and Sertoli cell proliferation.

The majority of postsmolt males had committed to sexual maturation on January 26, as indicated by the lower number of A_{und} spermatogonia and the higher number of A_{diff} and SPGB. Although a detailed morpho-functional evaluation of spermatogenesis in Atlantic salmon is still missing, studies on three other salmonid species have shown that spermatogonia go through six to eight rounds of mitotic divisions prior to entering meiosis (reviewed by Schulz & Nóbrega (2011)). Therefore, we can expect within 3 weeks a mitotic cell cycle every 2–3 days during the development from A_{und} to late SPGB in Atlantic salmon.

Androgen levels were further elevated at the end of January in the LD groups, tended to increase also in the LL groups, and *fshb* mRNA levels reached maximum values in a steep increase from the beginning of January. The steroidogenic activity of Fsh in fish has been discussed above. Classically, the production and release of gonadotropin would be stimulated by GNRH, acting via the GNRH receptor. While both pituitary *gnrhr4* and *fshb* mRNA levels were elevated in all groups on January 26, this was most prominently the case in the SW groups, as revealed by two-way ANOVA, and we interpret the higher number of SPGB found in these groups as reflecting stronger pro-differentiation signals (Fsh, androgen) stimulating spermatogenesis. Next to the salinity effect, it is possible that the shift to the shorter photoperiod provided an additional stimulus, perhaps via the GNRH neurons (Amano *et al*. 1999), which could explain the strongest increase in *fshb* transcript and plasma androgen levels in the SWLD group on January 26.

Future work will have to clarify the physiological mechanisms mediating SW-induced increases in pituitary *gnrhr4* transcript levels and the accelerated production of SPGB. Two observations are interesting to note in this context. First, hyperosmotic stress increased growth hormone-mediated, hepatic insulin-like growth factor 1 production (Meier *et al*. 2009), which may have increased pituitary *gnrhr* mRNA levels (Luckenbach *et al*. 2010). Secondly, hyperosmotic stress following SW exposure might also have stimulated vasopressin release (Balment *et al*. 2006), which in turn can directly stimulate testicular androgen release (Rodríguez & Specker 1991).

In general, spermatogenesis and steroidogenesis are regulated by gonadotropins in vertebrates. As has been discussed earlier, Fsh plasma levels are elevated in salmonid fish at the onset as well as during most of the rapid growth phase of the pubertal testis (Suzuki *et al*. 1988, Swanson *et al*. 1989, Oppen-Berntsen *et al*. 1994). The steep increase in *fshb* mRNA levels in all groups at the onset of puberty, an observation made in maturing salmon parr as well (Maugars & Schmitz 2008*a*), strongly supports the view that Fsh is the gonadotropin of major relevance for the onset of puberty. Primary tissue culture studies using immature Japanese eel testis suggested that spermatogonial proliferation and differentiation was solely mediated by the steroidogenic effect of Fsh (Ohta *et al*. 2007), but direct effects of Fsh on Sertoli cells are possible in other fishes. In zebrafish, Fsh downregulated Sertoli cell gene expression of anti-Müllerian hormone (*amh*) independent of sex steroid action (Skaar *et al*. 2011). As Amh inhibited both germ cell differentiation and steroid production (Skaar *et al*. 2011), Amh downregulation by Fsh is important for the sustained activity of the two main testicular functions. Downregulation of testicular *amh* mRNA levels was also reported from precociously maturing Atlantic salmon parr (Maugars & Schmitz 2008*b*). Finally, recent work in rainbow trout has shown that Fsh induced changes in testicular gene expression independent of its steroidogenic activity, including factors belonging to paracrine regulatory pathways (Sambroni *et al*. 2013).

Lh plasma levels usually increase in salmonid fish when approaching the spawning season after most of the testicular growth has been achieved (Gomez *et al*. 1999, Campbell *et al*. 2003). Still, *lhb* mRNA levels increased gradually during spermatogenesis, similar to previous findings in maturing male Atlantic salmon parr (Maugars & Schmitz 2008*a*) or in maturing Atlantic salmon female grilse (Andersson *et al*. 2013). This observation is probably related to the positive feedback effect of testicular steroids on pituitary *lhb* gene expression, being strong for testosterone and depending on its aromatization (Xiong *et al*. 1994, Antonopoulou *et al*. 2009). However, also the non-aromatizable androgen 11-KT had a (weaker) positive feedback effect on the pituitary Lh amount (Borg *et al*. 1998).

While the spermatogenic wave was developing faster initially in the two SW groups, this changed when in March, irrespective of the salinity, the LD groups developed faster toward completion of the wave. This was evident by the earlier decrease in the numbers of SPGB and spermatocytes and the earlier increase in the number of spermatozoa, showing that the production of new spermatogenic cysts had stopped earlier and that existing cysts completed their development earlier in the LD than in the LL groups. The males exposed to LD moreover showed 11-KT levels twice as high as the LL groups.

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Based on the pituitary gene expression data, in particular with regard to the *gnrhr4* and *lhb* transcript levels, and in the light of previous work on elevated Lh blood levels during spermiation, we propose that the stronger increase in androgen plasma levels in these groups reflects elevated Lh secretion. The short photoperiod may have activated the GNRH–Lh–androgen axis (Amano *et al*. 1999, 2001). It has not been clarified yet whether the additional increase in androgen levels in fully mature male salmonids is required for spermiation or can be understood in the context of secondary sexual characteristics or reproductive behavior. If acting on the testis, it may include stopping the production of differentiating spermatogenic cysts. In stickleback, prolonging the period with high androgen plasma levels postponed the restart of spermatogenesis for the next reproductive season (Andersson *et al*. 1988). However, stopping the production of differentiating spermatogenic cysts might also involve the downregulation of *fshb* mRNA (Fig. 5b) and Fsh plasma levels (Gomez *et al*. 1999). After all, downregulation of *fshb* mRNA levels can be induced by high androgen doses in Atlantic salmon parr (Antonopoulou *et al*. 2009).

Taken together, this study shows that Atlantic salmon postsmolts can reach sexual maturation under different environmental conditions and provides evidence for a differential activation of the brain–pituitary–testis axis, where the start of puberty is enhanced (via GNRH-Fsh-androgens) by SW and completion of puberty (via GNRH-Lh-androgens) by short days. Hence, although salinity and photoperiod conditions clearly modulated the response, the overall result of high maturation under elevated temperatures suggests that exposure to long days at the end of the smoltification regime in combination with relatively high water temperature may be sufficient to trigger maturation.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/JOE-13-0240.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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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 spermatogonia.

1. Introduction

Pubertal gonad maturation in vertebrates usually requires stimulation by the pituitary gonadotropins luteinizing hormone (Lh) and follicle-stimulating hormone (Fsh). In salmonid fish, however, Fsh is the only gonadotropin available in the blood during the onset of puberty and increased pituitary and plasma Fsh levels were associated with elevated androgen levels and spermatogonial proliferation and differentiation activity (Melo et al., 2014; Maugars and Schmitz, 2008; Campbell et al., 2003; Gomez et al., 1999). The main androgen produced by fish testes is 11-ketotestosterone (11-KT) (Borg, 1994) but significant concentrations also of testosterone (T) are found in the blood of salmonid species (e.g. Freeman et al., 1983; Liley et al., 1986; Scott and Sumpter, 1989), and both T and 11-KT are involved in the feedback control of reproduction in salmonids (e.g. Antonopoulou et al., 1999; Goos et al.,

Abbreviations: 11-KT, 11-ketotestosterone; Amh, anti-Müllerian hormone; Fsh, follicle stimulating hormone; gnrhr4, gonadotropin-releasing hormone receptor 4; Igf3, insulin-like growth factor 3; Lh, luteinizing hormone; OA, adrenosterone; T, testosterone.

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1986). 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*; Sambroni 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 spermatogonia, 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 (Sambroni et al., 2013a). However, a quantitative evaluation of androgen-induced germ cell development in combination with quantifying testicular mRNA levels of *amh* and *ifg3* 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 *gnrhr4* 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.

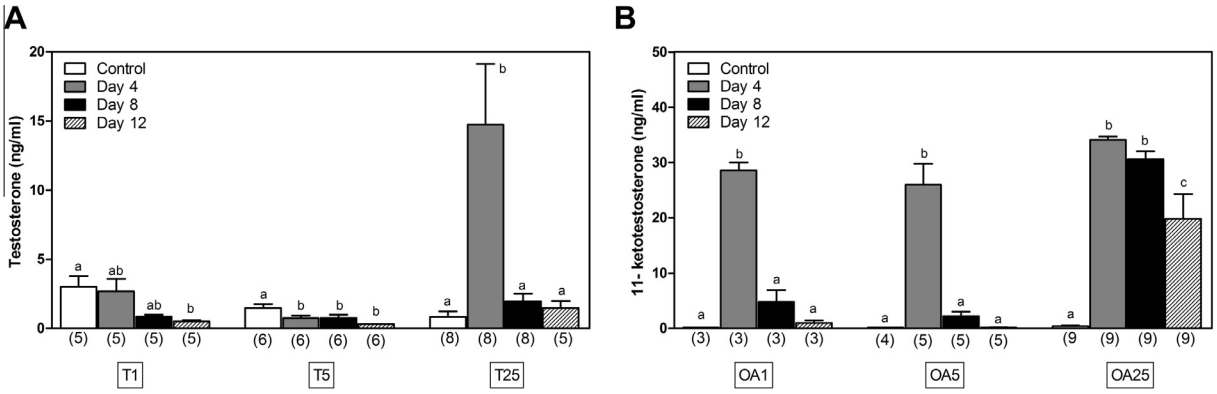


Fig. 1. Plasma androgen levels of male Atlantic salmon treated with different androgen doses in a dose-finding pilot experiment. Testosterone (A) and 11-Ketotestosterone (B) plasma levels after 4, 8 and 12 days of androgen injection. Bars show mean values ± SEM for the three different concentrations used (1, 5, or 25 μg/g body weight): T(1), T(5), T(25) and OA(1), OA(5), OA(25). Different lower case letters denote means that are significantly different time-wise ($P < 0.05$; one-way ANOVA followed by SNK test). The number of males per group is indicated between brackets under the respective bars.

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://imagej.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 (A_{und}; 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 (A_{diff}; 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 A_{diff}). 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 (pituitaries) 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 (Bogerd 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 *ara2* [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,

Table 1
Forward and reverse primers, and TaqMan fluorogenic (FAM-TAMRA) probes, used in real-time, quantitative PCR assays to determine the relative *amh* and *igf3* mRNA levels in Atlantic salmon pituitaries and gonads.

Target	Accession number	Primer name	Sequence 5′→3′	Slope	R^2
amh	AY722411	Fw	CAGTCACTCTCTGCAGCCTTACAA	3.3265[a]	0.9917
		Rv	CAACATTGAATCTCCATTTCAGTTTAC		
		Probe	TTTGCCCTCGGGTTGCTTTCCTGTT		
igf3	JN635414	Fw	GACCGACCGACAAGATGCA	2.111[b]	0.9929
		Rv	GCAAGGCACAATATGGAGTACA		
		Probe	AACATAGCACCTTCAGTGTGCATCCTCTGG		

[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.

Table 2
Primers used in Ar cloning study.

Primer name[a]	Sequence 5′→3′
1432 (Fw1; 3'-RACE)	CCCTCGGAGCACGTAAACTGAAGAAGATTGGACA
1433 (Fw2; 3'-RACE)	GACTTTCCATTCCCAGCTGGTGTTCCTCAA
1434 (Rv1; 5'-RACE)	AGAGATACACTGGATGGCATCAGTGGGGC
1435 (Rv2; 5'-RACE)	TTCAAAACACTTCCTCAGTCGACATGATGG
1512 (Fw; PCR)[b]	TA*GCCACC*ATGGAGATTCCAGTTGGATTAGGRGG
1513 (Fw; PCR)[b]	TA*GCCACC*ATGCGCCAAACTAACCGGCAATCACCC
1514 (Rv; PCR)	TTGCTACTTGTGGAAAAGGATTGGTTTGGC
1515 (Rv; PCR)	TTATCATGTGACTAAAGAACACCAAATAAGGAAA

[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.

Netherlands) to generate *ara1* and *ara2* expression vector constructs, of which the inserts were sequence verified.

Multiple sequence alignments of several androgen receptor deduced amino acid sequences were generated with Clustal W software (Thompson et al., 1994) using default settings. A phylogenetic tree was realized with the same software, including bootstrap analysis for 1000 iterations. The species from which full-length (F) or partial (P) androgen receptor sequences were obtained and the respective accession numbers are listed in the legend to Fig. S1, showing the cladogram of vertebrate androgen receptors.

HEK293T cells were co-transfected with 500 ng *ara1* or *ara2* expression vector construct in combination with 10 μg of MMTV (mouse mammary tumor virus)-luciferase plasmid. One day later, the transfected cells were transferred to 24-well plates coated with poly-L-lysin hydrobromide (Sigma–Aldrich, Zwijndrecht, Netherlands). The next day, the medium was replaced with transactivation assay medium (DMEM without phenol red, supplemented with charcoal-stripped FBS, glutamine and non-essential amino acids) containing steroids at the appropriate concentrations. After 24–36 h, cells were harvested in lysis mix (100 mM potassium phosphate [pH 7.7], 1% Triton X-100 [Sigma–Aldrich, Zwijndrecht, Netherlands], 15% glycerol and 2 mM dithiothreitol) and the luciferase activity was determined by adding an equal volume of substrate mix (100 mM potassium phosphate [pH 7.7], 250 mM beetle luciferin, 1 mM dithiothreitol, 2 mM adenosine triphosphate and 15 mM magnesium sulfate [Promega, Leiden, Netherlands]) before measuring luminescence (PerkinElmer luminometer).

To study steroid hormone binding to both Ar subtypes, HEK293T cells transfected with either the *ara1* 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® 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 experiment, and K_i values were calculated using the formula: $K_i = (IC_{50\text{-}steroid}/IC_{50}testosterone)^*K_d$[^{3}H]-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 treatment 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 μg/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-treated group). However, 14 days after injection of T, the T plasma

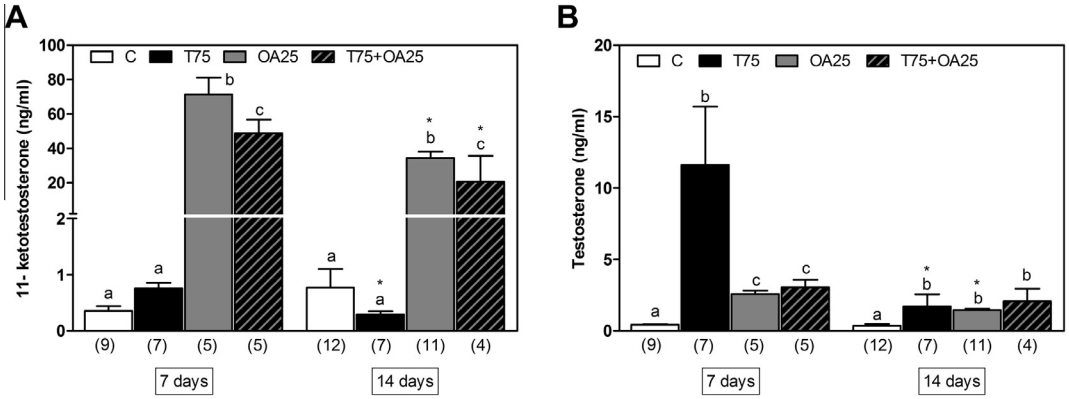


Fig. 2. Plasma androgen levels of 11-Ketotestosterone (A) and testosterone (B) in male Atlantic salmon 7 and 14 days after androgen injection. Bars show the mean values ± SEM from four treatment groups: control (C), T at 75 μg/g body weight (T75), OA at 25 μg/g body weight (OA25), and the combination 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 spermatogonia type A_{und} in these testes (Fig. 3B, see inset for an example of the morphology of type A_{und} spermatogonia) 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 A_{und} and type A_{diff} spermatogonia 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 A_{diff} spermatogonia, 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

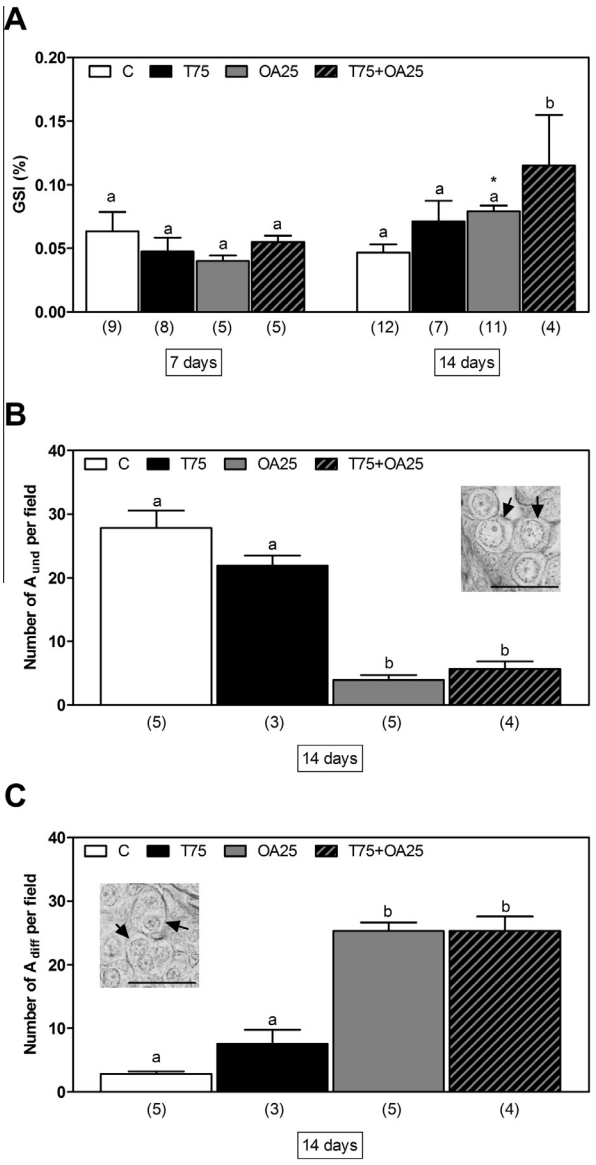


Fig. 3. Androgen effects on the gonado-somatic index (GSI; A) and the number of undifferentiated (A_{und}; B) and differentiating spermatogonia (A_{diff}; C) in Atlantic salmon. Bars show means ± SEM from four treatment groups: control (C), T at 75 μg/g body weight (T75), OA at 25 μg/g body weight (OA25), and the combination of the two androgens (T75 + OA25). Arrows in the histology micrographs indicate cells showing the typical morphological characteristics of A_{und} (inset in B) or A_{diff} (inset in C) spermatogonia. Scale bar in (B) and (C) = 25 μm. Different letters denote significant differences between treatment groups within a sampling date. ($P < 0.05$; one-way ANOVA followed by SNK test). Asterisks above the bars denote samples 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.

the presence of cysts with type A_{diff} spermatogonia 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 similarly low GSI values showed that the testes contained mainly A_{und} spermatogonia (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 A_{diff} 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 A_{und} into A_{diff} spermatogonia, the (self-renewal) proliferation of A_{und} was not stimulated, because the reservoir of A_{und} germ cells became depleted. Apparently, other or additional signals are required to stimulate the self-renewal proliferation of spermatogonia type A_{und}, 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, minor and statistically not significant changes in the numbers of spermatogonia type A_{und} and type A_{diff} were recorded (Fig. 3B and C). These observations allow two conclusions: (i) the *amh* transcript levels required more time to climb 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 A_{und}; 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*

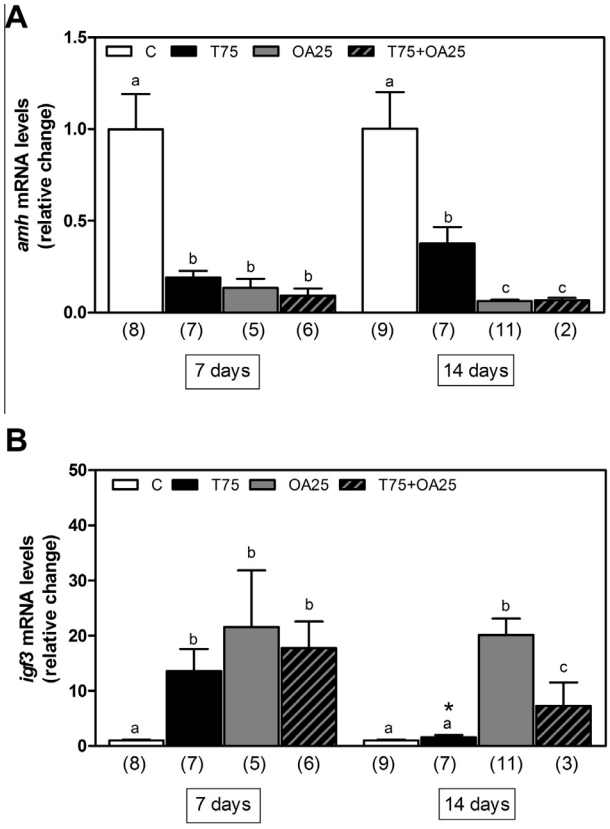


Fig. 4. Androgen effects on relative changes of testicular *amh* (A) and *igf3* (B) mRNA levels in Atlantic salmon 7 and 14 days after androgen injection. Bars show the mean values ± SEM from four treatment groups: control (C), T at 75 μg/g body weight (T75), OA at 25 μg/g body weight (OA25), and the 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.

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; Antonopoulou 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

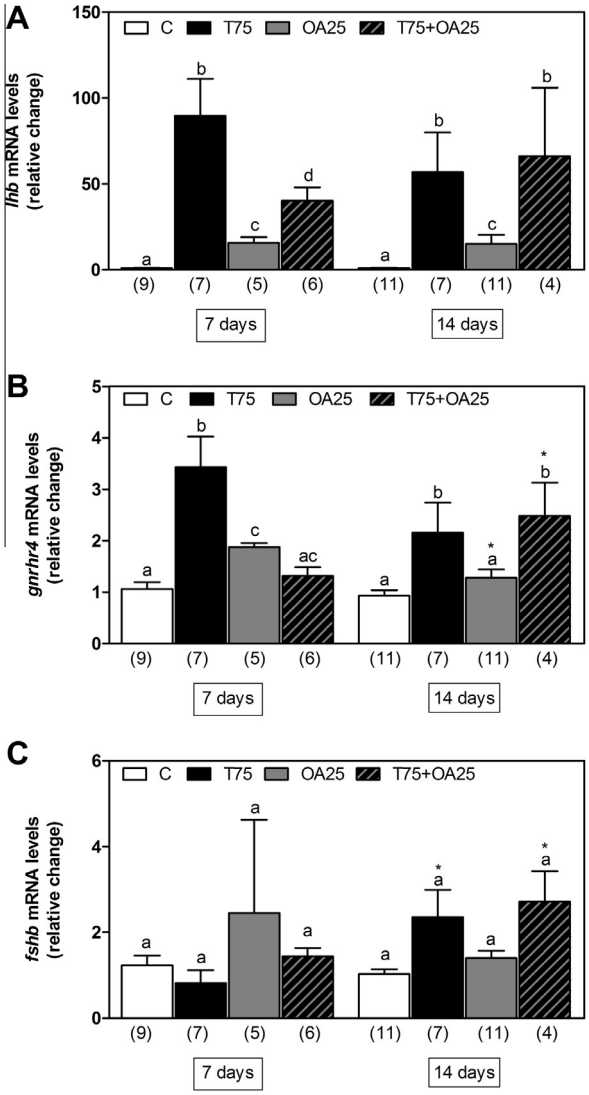


Fig. 5. Relative pituitary mRNA levels of the *lhβ* (A), *gnrhr4* (B) and *fshb* (C) genes in Atlantic salmon postsmolts 7 and 14 days after androgen injection. Bars show means ± SEM from four treatment groups: control (C), T at 75 μg/g body weight (T75), OA at 25 μg/g body weight (OA25), and the combination 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.

observations and the concept that aromatizable androgens exert a positive feedback on *lhb* transcript levels in the teleost pituitary.

The *lhb* mRNA levels in fish treated with OA were also significantly higher than in the control group but remained ~3- to 5-fold lower than in fish treated with T (Fig. 5A). Increases in *lhb* mRNA levels after treatment with OA or 11-KT have also been reported in experiments with stickleback (Hellqvist et al., 2008), catfish (Cavaco et al., 2001), and salmon (Antonopoulou et al., 1999). Since OA and 11-KT cannot be converted to estrogens, a molecular mechanism will be involved that does not depend on estrogen receptors. When castrated 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 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).

3.3.2. *fshb*

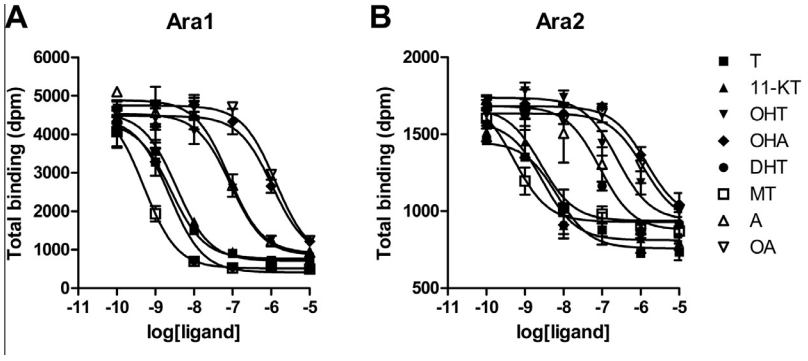
In salmonids, the start of spermatogenesis is accompanied by increased pituitary *fshb* mRNA and serum 11-KT levels (Melo et al., 2014; Maugars and Schmitz, 2008; Campbell et al., 2003; Gomez et al., 1999). The steroidogenic activity of Fsh in salmonid males (Planas et al., 1993; Planas and Swanson, 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 A_{und} 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 new spermatogenic cysts; also a decrease in the androgen sensitivity of the brain-pituitary system may play a role as permissive factor in allowing Fsh release at the beginning of puberty despite elevated androgen plasma levels. After all, elevated plasma levels of Fsh and 11-KT have repeatedly been recorded during the period of superallometric testis growth in salmonids (e.g. Gomez et al., 1999; Campbell et al., 2003).

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 Ara 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 (K_d of 1.6 ± 0.1 nM) and 11-KT (K_d of 1.7 ± 0.1 nM) than Ara2 (T K_d of 9.2 ± 0.6, 11-KT K_d of 4.5 ± 0.1 nM). Using [^{3}H]-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 K_i 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)



C

	Ara1			Ara2		
T (Kd)	1,6	±	0,1	8,2	±	0,6
11-KT	4,5	±	1,3	12,8	±	4,6
OHT	56,3	±	6,5	525	±	146
OHA	589	±	45	626	±	207
DHT	0,9	±	0,3	4,1	±	1,4
MT	0,3	±	0,1	1,5	±	0,6
A	44,2	±	8,3	131	±	56
OA	395	±	214	811	±	291

Fig. 6. Androgen binding to both androgen receptors (Ar) subtypes expressed in HEK293T cells transfected with either the Ara1 (A) or the Ara2 (B) expression vector. Curves show the mean values ± SEM of dose–response studies from one experiment done in duplicate. The numbers given in (C) show the K_i values ± SEM from three independent such dose–response experiments. T Testosterone (T), 11-ketotestosterone (11-KT), 11β-hydroxytestosterone (OHT), 11β-hydroxyandrostenedione (OHA), 5α-dihydrotestosterone (DHT), methyltestosterone (MT), androstenedione (A) and adrenosterone (OA). The K_d concentration for T has been determined from three independent transfections (see Fig. S2).

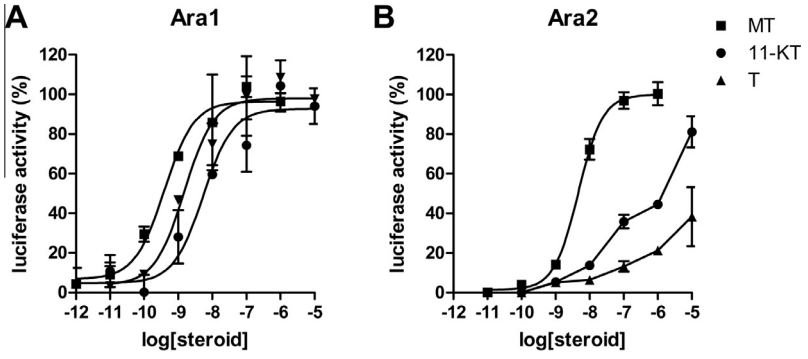


Fig. 7. Androgen specificity of the transcriptional activity of Ara1 (A) and Ara2 (B). Symbols give means ± SEM of dose–response studies from one experiment done in duplicate relative to the maximal luciferase activity induced by each androgen at various concentrations, where the most active androgen, methyltestosterone (MT), is represented as 100%; 11-ketotestosterone (11-KT) and testosterone (T).

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 EC_{50} 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 salmonids (Douard 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 or maintaining (too) 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.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ygcen.2014.11.015.

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4 - PERSPECTIVAS

E

CONCLUSÕES

Os resultados obtidos no capítulo 2 do presente estudo mostraram que o salmão do Atlântico no estágio *postsmolt* pode atingir a maturidade sexual sob diferentes condições ambientais. Assim, os dados encontrados forneceram importantes evidências morfofisiológicas acerca da ativação da via hipófise-testículo, a qual se alterou de acordo com os tratamentos impostos, onde a salinidade exerceu efeitos estimulatórios nas fases iniciais da puberdade, enquanto o rápido progresso da espermatogênese foi coordenado pelo fotoperíodo curto, possivelmente através de ação dos andrógenos. Contudo, apesar da salinidade e fotoperíodo ter claramente modulado a resposta fisiológica, a alta taxa de maturação sexual sob temperatura elevada sugere que a exposição dos salmões a dias longos no fim do regime de esmoltificação, associado com temperatura relativamente mais alta, pode ser suficiente para estimular a maturação sexual. Entretanto, futuros experimentos serão necessários para identificar as vias endócrinas ativadas pela salinidade, bem como para comprovar se o efeito estimulatório propiciado pelo fotoperíodo ocorreu através dos andrógenos.

Já os resultados obtidos no capítulo 3 mostraram que o tratamento de peixes sexualmente imaturos com os esteróides sexuais 11-cetotestosterona e testosterona modulou a expressão de fatores de crescimento testicular que, quando mantida por 2 semanas, estimulou a diferenciação, mas não auto-renovação, das espermatogônias indiferenciadas. Contudo, a atividade biológica dos esteróides não foi mediada pelos receptores de andrógenos, uma vez que estes últimos não foram capazes de diferenciar os andrógenos. Assim, este aspecto merece particular atenção em investigações posteriores.