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

Journal of Endocrinology
(2014) 220, 319–332

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

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 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 psmolts were distributed over 16 experimental tanks of 500 l with 100 fish per tank (body weight 151 ± 19 g (mean $\pm$ 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.

January 12 and was maintained at $15.5 \pm 1.1^\circ\text{C}$ 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 $\text{GSI (\%)} = \text{gonad weight (g)} \times 100 / \text{total body weight (g)}$. 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 $5\ \mu\text{m}$ 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 $600\times$ 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 $\sim 15\ \mu\text{m}$), differentiated type A spermatogonia (SPGA_{diff}; two or more cells with some heterochromatin and a nuclear diameter of $\sim 10\ \mu\text{m}$), and type B spermatogonia (SPGB; groups of cells with a high amount of heterochromatin and a nuclear diameter

below $8\ \mu\text{m}$). 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 $5\ \mu\text{m}$ 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 $1000\times$ 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 IPrep™ 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 $2\ \mu\text{g}$ 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.

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 (%)
<i>gnrhr4</i>	KF225730	Fw	TCAACCCACTGGCGATCAAT	101.8
		Rv	CGTGATGGTCACACTGTGGAATA	
		Pr	AGTGTGATTCTGTCTGTTCCCAGATGCTG	

All TaqMan assays were performed in 10 µl reaction volumes and their C_q 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 Dunnett'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 $\pm$ 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.

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 $\times$ 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.

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
<i>gnrhr4</i>	S, P	S, P	P	S, P, I
<i>fshb</i>	S, P	P	P, I	S, P
<i>lhb</i>	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 Dunnett'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

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 Dunnett'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

Figure 5

Relative pituitary mRNA levels of the *gnhr4* (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 Dunnett'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.

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 gonadotrophs 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.

Figure 7

Analyses of different parameters (11-ketotestosterone, 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

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 2008a), 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 2008b). 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 2008a) 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.

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.

Funding

This study was supported by the Norwegian Research Council (contract 199518) and Norwegian Ministry of Fisheries and Coastal Affairs (contract 12622-01), Utrecht University, and the Brazilian Foundation CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior).

Acknowledgements

The authors wish to thank the technical staff at the Institute of Marine Research, Matre for their excellent technical support.

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Received in final form 22 November 2013

Accepted 19 December 2013

Accepted Preprint published online 20 December 2013