

Cloning, pharmacological characterization, and expression analysis of Atlantic salmon (*Salmo salar* L.) nuclear progesterone receptor

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Abstract

To better understand the role(s) of progestogens during early stages of spermatogenesis, we carried out studies on the nuclear progesterone receptor (Pgr) of the Atlantic salmon. Its open-reading frame shows the highest similarity with other piscine Pgr proteins. When expressed in mammalian cells, salmon Pgr exhibited progestogen-specific, dose-dependent induction of reporter gene expression, with 17 α ,20 β -dihydroxy-4-pregnen-3-one (DHP) showing the highest potency. We then analyzed testicular *pgr* mRNA and DHP plasma levels in animals during the onset of spermatogenesis, which were exposed to natural light or to constant light, to induce significant differences in testis growth. Grouping of the animals according to their progress through spermatogenesis showed that testicular *pgr* mRNA levels as well as DHP plasma levels first increased when germ cells had reached the stage of late type B spermatogonia and further increased when entered meiosis, i.e. when spermatocytes were present. However, *in situ* hybridization studies revealed that *pgr* mRNA expression was restricted to Sertoli cells, with a strong signal in Sertoli cells contacting type A/early type B spermatogonia, while Sertoli cells contacting larger germ cell clones with further differentiated stages (e.g. late type B spermatogonia) were less intensely/not stained. We conclude that the increase in *pgr* mRNA levels per pair of testis reflects, at least in part, the increased number of Sertoli cells enveloping type A and early type B spermatogonia. We propose that Sertoli cell-expressed Pgr may mediate DHP-stimulated early steps in spermatogenesis in Atlantic salmon, such as an increase in the number of new spermatogonial cysts.

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Introduction

Spermatogenesis is a cellular developmental process divided into three major phases: the spermatogonial phase with different generations of spermatogonia; the meiotic phase with primary and secondary spermatocytes; and the spermiogenic phase with haploid spermatids and spermatozoa (Schulz *et al.* 2010). Vertebrate spermatogenesis is controlled by the coordinated action of a range of hormones and growth factors. In teleost fish, also sex steroids of the progestin family, such as 17 α ,20 β -dihydroxy-4-pregnen-3-one (DHP) or 17 α ,20 β ,21-trihydroxy-4-pregnen-3-one (20 β -S), play important roles during spermatogenesis. For example, they induce spermiation (Ueda *et al.* 1985), increase milt production (Baynes & Scott 1985), and stimulate spermatozoa motility (Miura *et al.* 1992, Tubbs & Thomas 2008).

In salmonid fish, there are two peaks of DHP plasma levels during their reproductive cycle. The quantitatively more important peak is observed in the spawning season, while a smaller and transient increase is reported during the progression of spermatogonial proliferation (Dépêche & Sire 1982, Scott & Sumpter 1989, Vizziano *et al.* 1996), suggesting that DHP plays a role not only during spermiation and spawning but also during early stages of spermatogenesis. Indeed, DHP induced the initiation of meiosis in male germ cells in Japanese eel (*Anguilla japonica*; Miura *et al.* 2006).

The biological activity of progestogens is mediated via specific receptors. Nuclear and membrane-bound progestogen receptor types are highly expressed in fish testis (Ikeuchi *et al.* 2002, Hanna & Zhu 2009). Recent results suggest that membrane-associated progestin receptor α (mPR α) mediates the non-genomic actions of progestins

to induce sperm hypermotility in a number of teleost species (Tubbs & Thomas 2008). In Japanese eel, progesterone receptor 1 (*pgr1*) mRNA was expressed in germ cells, Sertoli cells, and interstitial cells of testis, whereas *pgr2* mRNA was detected only in germ cells (Miura *et al.* 2006). In our previous work on zebrafish (*Danio rerio*), we found that *pgr* mRNA was expressed in Leydig and Sertoli cells (Chen *et al.* 2010). However, other data indicated that Pgr protein can be detected in germ cells (Hanna *et al.* 2010). Taken together, these data open the possibility that the Pgr may be involved in mediating DHP effects on early stages of spermatogenesis in teleost fish.

To broaden the data basis as regards the role of Pgr in fish spermatogenesis from eel (Anguilliformes) and zebrafish (Cypriniformes) to a third unrelated order, the Salmoniformes, we isolated a full-length *pgr* cDNA from Atlantic salmon (*Salmo salar* L.) and characterized pharmacologically the salmon Pgr. To investigate the possible role of Pgr during the spermatogonial proliferation phase, we determined the cellular localization of the *pgr* mRNA in salmon testis by *in situ* hybridization. Finally, making use of the fact that the onset of puberty in Atlantic salmon is sensitive to photoperiod manipulation, we analyzed changes in testicular *pgr* mRNA levels, plasma DHP concentrations, and testis histology during early spermatogenesis in fish exposed to photoperiod conditions that stimulated or inhibited the onset of pubertal testis growth.

Results

Isolation and sequence analysis of salmon *pgr* cDNA

The open-reading frame (ORF) of the salmon *pgr* consisted of 2157 nucleotides (GenBank accession number: GU583841), encoding a protein of 718 amino acids (Supplementary Figure 2, see section on supplementary data given at the end of this article). Comparison of the deduced amino acid sequence of the salmon *pgr* with PGRs from other species is shown in Supplementary Table 2 (see section on supplementary data given at the end of this article). The salmon Pgr amino acid sequence could be subdivided into four domains. An N-terminal transactivation domain (TAD) showed low homology (10.5–26.7%), while the putative DNA-binding domain (DBD) and ligand-binding domain (LBD) showed high homology (DBD, 83.3–97.2%; LBD, 64.7–85.3%), with PGRs of other vertebrates. The overall homology of salmon Pgr with PGRs from other species is 37.0–62.9%. A phylogenetic tree, constructed from the aligned amino acid sequences using the neighbor-joining method, revealed that the known PGRs are divided into three major clades (Supplementary Figure 3, see section on supplementary data given at the end of this article). The first clade consisted of fish Pgrs, the second clade contained avian, reptilian, and amphibian PGRs, and the last clade contained mammalian PGRs.

Steroid-specific transactivation of the salmon Pgr

To determine the steroid-dependent transactivation properties of the salmon Pgr, HEK 293T cells, which do not display endogenous PGR activity (Chen *et al.* 2010), were transfected with the pGL3-MMTV-Luc reporter construct alone or together with the salmon *pgr* expression vector construct. Next, transfected cells were stimulated with increasing concentrations of different steroid hormones. Dose-dependent, Pgr-mediated activation of the MMTV promoter was clearly shown for DHP and 20 β -S (Fig. 1A), the one with the lowest EC₅₀ value being DHP (17.9 \pm 0.2 nM). Also at a fixed concentration of 1 μ M, DHP and 20 β -S were the most potent inducers of luciferase activity (four- and twofold above control respectively; Fig. 1B). The other three progesterone-related hormones tested elicited increases in luciferase activity only at a concentration of 10 μ M, while other steroid hormones assayed (testosterone, 11-ketotestosterone (11-KT), 17 β -estradiol (E₂), or cortisol) were ineffective at 10 μ M.

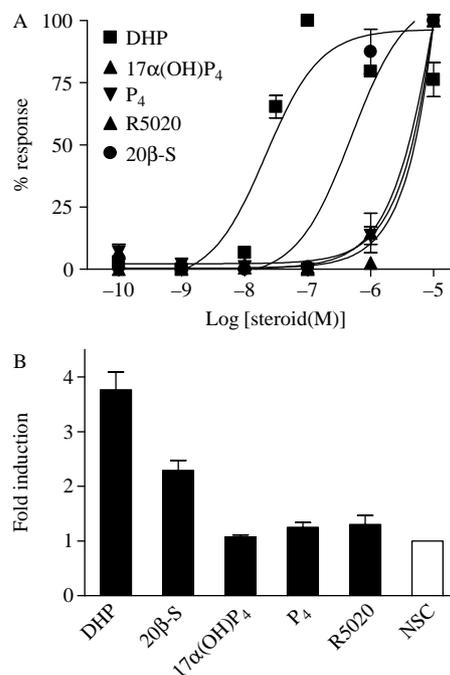


Figure 1 Ligand-induced transactivation properties of the Atlantic salmon Pgr. HEK 293T cells were transiently co-transfected with the pGL3-MMTV-Luc and the salmon *pgr* expression vector constructs. (A) Transfected cells were incubated with increasing concentrations (from 0.1 nM to 10 μ M) of various progesterones. Percentage (%) of response: values are given relative to the maximal amount of luciferase activity for each condition. Each point represents the mean \pm S.E.M. of three independent experiments, with duplicates for each steroid concentration. Curves were generated using non-linear regression (GraphPad Prism 4.0). (B) Transfected cells were incubated with or without 1 μ M of the steroids indicated. Data are expressed as the ratio of steroid:NSC (no steroid control). Each column represents the mean of three independent experiments, with the vertical bar representing the S.E.M.

Tissue distribution of salmon *pgr* mRNA

Real-time, quantitative PCR analysis of several tissues from immature animals showed that testicular tissue had the highest levels of *pgr* expression, followed by ovarian tissue. Some *pgr* mRNA expression was also found in the pituitary and spleen, while *pgr* mRNA levels were very low or undetectable in the other tissues tested (Fig. 2).

Cellular localization of *pgr* expression in salmon testis

Identification of cell types expressing salmon *pgr* mRNA in the testis at the onset of spermatogenesis was accomplished by *in situ* hybridization using testis cryosections. A strong signal was observed in particular in the perinuclear area of Sertoli cells surrounding early generations of spermatogonia (Fig. 3A and B). No signal was observed when adjacent sections were hybridized with the sense cRNA *pgr* probe (inset Fig. 3B). Sertoli cells surrounding type A and early type B spermatogonia were more intensely labeled than Sertoli cells associated with further developed germ cell types (Fig. 3C and D).

Effect of constant light on gonadosomatic index and testis histology

All males kept under natural light (NL) conditions were recruited into maturation during the first half of the year. Hence, immature males with low gonadosomatic index (GSI) values and only type A spermatogonia were no longer found after 19 March (Supplementary Figure 4,

see section on supplementary data given at the end of this article). Males with testes showing early type B spermatogonia or late type B spermatogonia as the most advanced germ cell type were found on 18 February or 19 March and 25 April respectively, while spermatocytes were found in all males sampled on 11 June (Supplementary Figure 4). When sorted according to the most advanced stage of germ cell development, irrespective of the time of sampling, GSI values increased significantly with the progress in spermatogenesis (Fig. 4A; left of dashed line).

There were two main effects of exposure to constant light (LL). First, immature males showing only type A spermatogonia and low GSI values were found on all sampling dates, indicating that entry into maturation was blocked by exposure to LL for these fish, constituting ~40% of the LL-exposed males (Supplementary Figure 4). Second, in males maturing under LL conditions (~60% of LL-exposed males), testis growth exceeded the one found under NL conditions once the rapid growth phase had started after the sampling in April (Fig. 4A and B; Supplementary Figure 4). A comparison of GSI values between animals sampled in February and March showing early and late type B spermatogonia respectively revealed that irrespective of the light conditions, the first increase in GSI values was associated with the appearance of late type B spermatogonia (Fig. 4A and B; right of dashed line).

Analysis of plasma DHP levels and testicular *pgr* mRNA expression in relation to testis histology

When NL-exposed animals were grouped according to the stage of germ cell development, the DHP plasma levels were low when only type A spermatogonia were present in the testis but increased with progressing spermatogenesis (Fig. 4C). Comparing DHP levels in animals sampled in February or March that presented early or late type B spermatogonia respectively showed that the first increase in plasma DHP level was associated with the appearance of late type B spermatogonia (Fig. 4C, right of dashed line). A further increase was observed when spermatocytes were found in the testes (Fig. 4C and D; left of dashed line).

First, we calculated testicular *pgr* expression levels after normalization to elongation factor 1 α (*ef1 α*) mRNA levels but without applying corrections for testis or body size changes (Fig. 4E and F). In animals exposed to NL conditions, *pgr* mRNA levels were stable in testes containing type A and type B spermatogonia but decreased when meiosis had started (Fig. 4E; left of dashed line). Different from plasma DHP and GSI values, *pgr* expression did not differ between animals sampled in February and March showing early and late type B spermatogonia respectively (Fig. 4E; right of dashed line). However, when corrections for RNA extraction and testis and body weight changes were applied

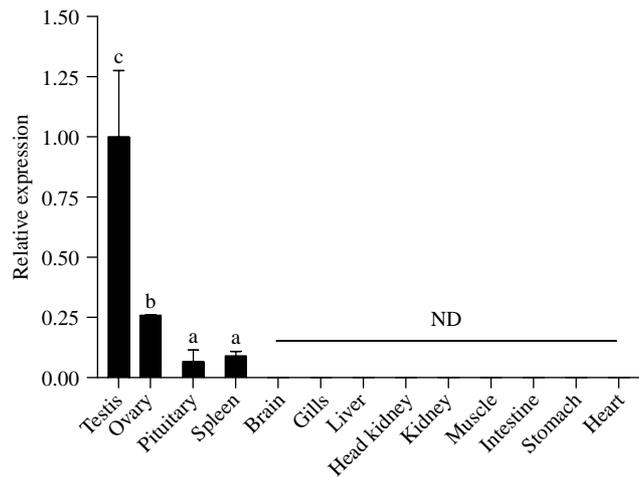


Figure 2 Relative expression of *pgr* mRNA in immature testis and in different tissues of immature female Atlantic salmon. The *pgr* mRNA expression levels were normalized to the expression of *ef1 α* mRNA. Values represent mean \pm S.E.M. ($n=3$) relative to testicular *pgr* mRNA levels. Tissues were collected at the beginning of December with GSI values below 0.05% in males and below 0.2% in females, as is typical for testes containing early spermatogonia, and typical for ovaries containing previtellogenic oocytes. Bars marked with different letters are significantly different from each other ($P<0.05$). ND, not detectable.

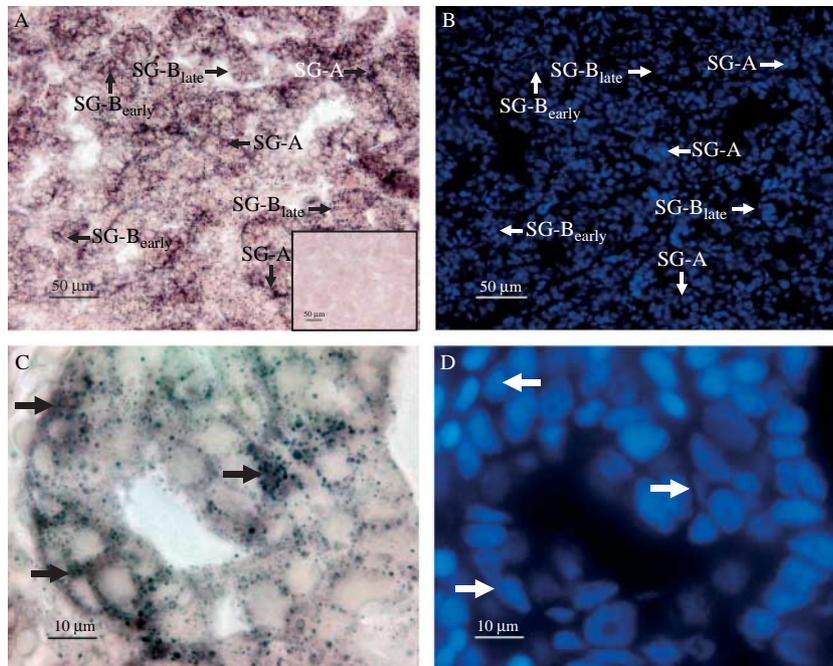


Figure 3 *In situ* hybridization of salmon *pgr* mRNA in testis from an NL-exposed male at the beginning of spermatogenesis, containing type A, early type B, and late type B spermatogonia. The antisense cRNA probe (A) strongly labeled Sertoli cells contacting type A (SG-A) and early type B spermatogonia (SG-B_{early}) that are recognized by the larger cell size and cysts containing single cells, pairs, or small groups of spermatogonia (B, DAPI-counterstained section). A weak label was found on Sertoli cells contacting late type B spermatogonia (SG-B_{late}). Note that interstitial tissue remained unstained. A higher magnification (C) shows the signal in the cytoplasm of Sertoli cells that are recognized by the triangular or elongated shape of their nuclei (D, DAPI-counterstained section). Black arrows (C) indicate Sertoli cell cytoplasm. White arrows (D) indicate Sertoli cell nuclei. Inset in (A) showed the sense cRNA probe that did not result in specific staining.

according to Kusakabe *et al.* (2006), the pattern of testicular *pgr* mRNA expression was similar to those of GSI and plasma DHP levels (Fig. 4G), i.e. a steady increase in *pgr* expression that followed the progress of germ cell development.

Grouping of the LL-exposed animals according to the stage of spermatogenesis resulted in patterns of plasma DHP and *pgr* mRNA expression levels (Fig. 4D, F, and H) that were very similar to those observed under NL conditions (Fig. 4C, E, and G). It seems, therefore, that for the changes in hormone level and its nuclear receptor, the developmental stage is a rather important parameter.

Discussion

The structural features of the cloned salmon *pgr* cDNA suggest that it encodes a member of the nuclear receptors family. These modular proteins are composed of a variable TAD at the N-terminus, a highly conserved DBD, a hinge region, and a conserved LBD at the C-terminus (Evans 1988). The salmon Pgr protein shares structural features with Pgr proteins from other teleost species. For example, the highly conserved DBD contains cysteine residues, constituting the two zinc finger motifs, as well as the P box (GSCKV) and the D box (AGRND) sequences (Umesono & Evans 1989), important regions for the recognition of Pgr target gene sequences, are all conserved in the salmon Pgr. The result of our comparative analysis of Pgr amino acid sequences was congruent with the phylogenetic relationship among the major vertebrate clades (Carroll 1988). This includes a proline-rich motif in the

N-terminal domain of the human PGR, responsible for the interaction with the c-Src family of tyrosine kinases (Boonyaratanakornkit *et al.* 2001), which is absent in salmon, zebrafish, and eel (Todo *et al.* 2000, Ikeuchi *et al.* 2002, Chen *et al.* 2010), so that this particular region may not be available for Pgr-mediated Mos/MAPK activation in teleosts.

For many genes, ray-finned fish have two paralogous copies, whereas one ortholog is present in tetrapods (Wittbrodt *et al.* 1998). This is related to the teleost-specific genome duplication that occurred after the split of the Acipenseriformes and the Semionotiformes from the lineage leading to teleost fish, but before the divergence of Osteoglossiformes (Hoegg *et al.* 2004). In eel, two distinct *pgr* genes have been reported (Todo *et al.* 2000, Ikeuchi *et al.* 2002). However, experimental trials to isolate additional *pgr* cDNAs or *in silico* approaches to identify related sequences did not provide evidence for the existence of additional *pgr*-like genes or mRNA isoforms from one gene in zebrafish, medaka, *Takifugu*, *Tetraodon*, and stickleback (Chen *et al.* 2010, Hanna *et al.* 2010). Our results from the phylogenetic analysis indicate that salmon and zebrafish Pgr as well as eel Pgr1 form a clade separate from eel Pgr2. Thus, it is likely that the salmon genome lost the additional *pgr* gene. Our experimental evidence obtained by extensive RT-PCR with multiple primer sets and *in silico* approaches further support the existence of a single full-length *pgr* transcript produced from a single locus in the salmon genome.

In mammals, birds, and amphibians, progesterone is considered to be the main ligand for their PGRs.

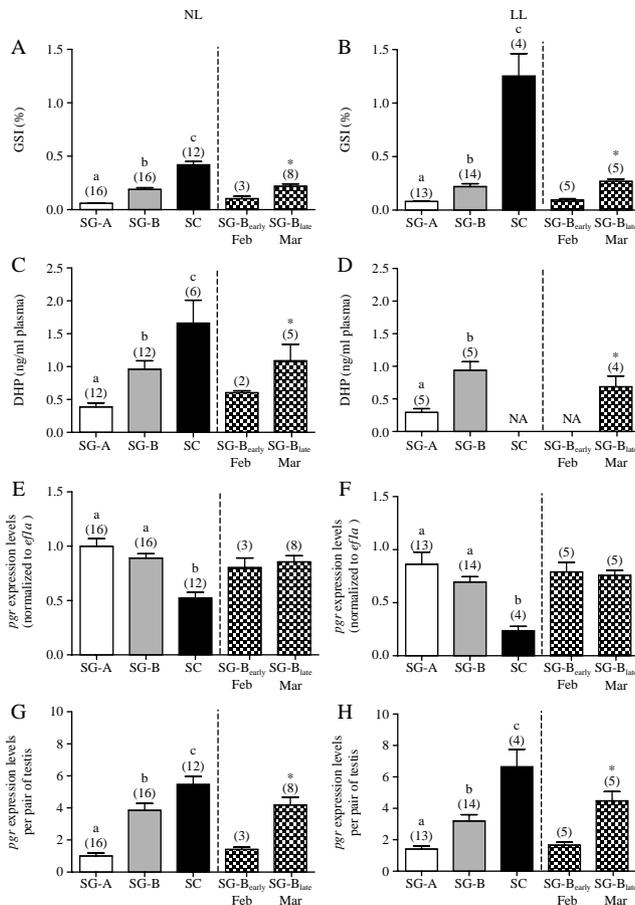


Figure 4 Changes in GSI (A and B), plasma DHP (C and D), and testicular *pgr* mRNA expression (E–H) levels in Atlantic salmon exposed to natural light (NL; A, C, E, and G) or to additional constant light (LL; B, D, F, and H) during the onset of spermatogenesis. To the left of the dashed line, males were assigned to groups based on the testicular histology, with abbreviations indicating SG-A (type A spermatogonia), SG-B (type B spermatogonia), and SC (spermatocytes). Bars marked with different letters are significantly different between each other ($P < 0.05$). To the right of the dashed line, data are shown of males sampled in February that showed early type B spermatogonia (SG-B_{early}) as the most advanced germ cell type in the testis, and of males sampled in March showing late type B spermatogonia (SG-B_{late}) as the most developed advanced germ cell type; in February, all males in the SG-B stage showed early type B spermatogonia, while all males in the SG-B stage in March and April showed late type B spermatogonia. Asterisks indicate significant differences to the mean values of groups showing SG-A or SG-B_{early} ($P < 0.05$). The number of animals per group is indicated between brackets above each column. NA, sufficient volumes of plasma samples were not available from some individuals.

In teleost fish, however, DHP is the main ligand for Pgrs (Ikeuchi *et al.* 2002, Chen *et al.* 2010). In this study, we demonstrated that the salmon *pgr* cDNA codes for a functional Pgr, which is able to transactivate target genes in a progestogen-dependent manner. Transactivation was progestogen specific, and DHP was the most effective steroid, supporting the view that DHP is the major native ligand for the salmon Pgr.

In contrast to the wide expression pattern of PGRs in mammals (Graham & Clarke 1997), the salmon *pgr* has a less broad expression pattern, which has also been observed in zebrafish and eel (Ikeuchi *et al.* 2002, Chen *et al.* 2010). However, in all vertebrates, PGRs are expressed predominantly in the reproductive organ (Graham & Clarke 1997, Wang *et al.* 2004, González-Morán *et al.* 2008). Interestingly, *pgr* mRNA has a less broad expression pattern than the androgen receptor in male teleosts (de Waal *et al.* 2008), although both receptor types were created from a 3-ketogonadal steroid receptor by the third genome duplication during vertebrate evolution (Thornton 2001). However, androgens have multiple functions in the morphological specification of the male phenotype that has evolved also in the teleost lineage, while Pgr-mediated functions seem restricted to reproductive physiology (Ogino *et al.* 2004). Ohno (1970) proposed that gene duplications facilitate the functional diversification of genes and generates the developmental and morphological complexity during evolution. This difference in functional diversification between androgen receptor and PGR may explain the presence of two subtypes of *pgr* genes only in eel, while distinct paralogous copies of androgen receptors have been identified in several species (Ogino *et al.* 2009).

The localization of PGRs in the vertebrate testis provided different results. Studies on boar, rat, and dog reported PGR protein localization to germ cells (Galena *et al.* 1974, Sirivaidyapong *et al.* 2001, Kohler *et al.* 2007). In the human testis, also Sertoli and Leydig cells were PGR-positive (Shah *et al.* 2005). However, a much more restricted distribution, namely to peritubular cells and to Leydig cells of human and non-human primate testes, was reported in a study using four different antibodies (Luetjens *et al.* 2006). In Japanese eel, *pgr1* mRNA was expressed in germ cells, Sertoli cells, and interstitial cells of testis, whereas *pgr2* mRNA was detected, by RT-PCR, only in germ cells (Miura *et al.* 2006). In zebrafish, we found *pgr* mRNA expression in Leydig and Sertoli cells only (Chen *et al.* 2010), while Hanna *et al.* (2010) reported Pgr protein expression in spermatogonia and spermatocytes. In this study, we found a strong *in situ* hybridization signal of salmon *pgr* mRNA in Sertoli cells surrounding type A and early type B spermatogonia, while Sertoli cells contacting larger clones with further differentiated germ cells (e.g. late type B spermatogonia) were less intensely/not stained. In Japanese eel, the molecular mechanism underlying DHP-mediated stimulation of spermatogenesis involved elevated expression of 11β -hydroxysteroid dehydrogenase and trypsinogen in Leydig and Sertoli cells respectively (Ozaki *et al.* 2006, Miura *et al.* 2009). The Sertoli cell expression that we report is consistent with the presence of Pgr in testicular somatic cells in vertebrates, but we could not detect *pgr* mRNA in Leydig cells. This is possibly related to the fact that our study was restricted to the initiation of spermatogenesis, a period during which

Leydig cell activity is still relatively low and plasma androgen levels are far away from their annual maximum values in the spawning season (Mayer *et al.* 1990).

In seasonally breeding teleosts, the size and cellular composition of the testis change considerably, which involves proliferation of germ cells and of somatic cells (Schulz *et al.* 2010). Moreover, testis growth is accompanied by a significant change in the amount of total RNA per testes (Kusakabe *et al.* 2002), and germ cell proliferation greatly exceeds Sertoli cell proliferation (see below), so that the proportion of somatic cell transcripts in the total testis mRNA becomes under-represented when spermatogenesis progresses. Based on this point of view, we understand the decrease in testicular *pgr* expression levels that is associated with the appearance of spermatocytes when normalizing the results to *ef1 α* only (Fig. 4E and F) as reflecting the dilution of Sertoli cell-derived mRNAs during germ cell proliferation.

Hence, a manner to report qPCR data for relative gene expression should be developed for an alternative presentation of the testicular expression data. Kusakabe *et al.* (2006) described an approach that incorporated RNA yield (μg RNA recovered per mg tissue extracted) and gonad and body weight changes into the calculation of the relative level of target gene expression, in addition to normalization to *ef1 α* mRNA levels. We have applied the correction for testis weight based on the following reasoning. *In situ* hybridization showed that *pgr* mRNA is restricted to Sertoli cells. The seasonal spermatogenesis/testis growth is associated with both Sertoli and germ cell proliferation in fish (Schulz *et al.* 2010), and Sertoli cell proliferation mainly occurs during the mitotic expansion of the spermatogonial population (e.g. Schulz *et al.* 2005). However, germ cell proliferation greatly exceeds Sertoli cell proliferation, so that the germ/Sertoli cell ratio increases from 0.5 to 0.8 in cysts containing single type A spermatogonia to ~ 100 –170 in cysts containing spermatids (Billard 1969, Matta *et al.* 2002, Schulz *et al.* 2005, Leal *et al.* 2009). Data on germ cell and Sertoli cell proliferation are not available for salmon, but histological analysis of salmonid testes suggests that germ cell proliferation greatly exceeds Sertoli cell proliferation also in salmonids (e.g. Billard 1983, Schulz 1984). Hence, the cellular composition of maturing testis changes such that Sertoli cell-derived mRNAs become progressively diluted by germ cell mRNAs up to a factor of ~ 125 (i.e. $100/0.8$). Also the GSI increases ~ 100 -fold in males in different seasonally reproducing species (e.g. Schulz 1984, Almeida *et al.* 2008), again mainly reflecting an increase in germ cell number. Therefore, testis mass changes are suitable to estimate the dilution of Sertoli cell-derived mRNAs, in particular up until the end of meiosis, since spermatogonia and spermatocytes contain normal and high amounts of mRNAs respectively while this is different once the relative masses of spermatids and spermatozoa increase, since

haploid cells contain little mRNA. However, except for two fish in the final sampling showing the first spermatids, these later maturational stages were not present in this study. The correction for body weight has been applied to take into account interindividual, isometric differences in testis weight. When calculated according to Kusakabe *et al.* (2006), *pgr* mRNA expression levels increased progressively during early spermatogenesis (Fig. 4G and H). Since *in situ* hybridization results indicated that expression of *pgr* mRNA was most prominent in Sertoli cells contacting type A and early type B spermatogonia, we conclude that the progressive increase in testicular *pgr* mRNA levels reflects the increased number of new spermatogonial cysts formed during the onset of testis growth, or more specifically, the increased number of Sertoli cells enveloping the newly formed type A and early type B spermatogonia.

One of the aims of this study was to investigate whether DHP, via a Pgr-mediated pathway, could participate in stimulating early spermatogenesis, by examining changes in *pgr* mRNA expression and plasma DHP during the onset of puberty in Atlantic salmon. A function of DHP during early spermatogenesis was first suggested by Dépêche & Sire (1982), who reported that rainbow trout testis tissue produced DHP from $17\alpha(\text{OH})\text{P}_4$ when testis growth began with the start of rapid spermatogonial proliferation. Using a primary testis tissue culture system, it was further demonstrated that DHP induced spermatogonial DNA synthesis in Japanese huchen and eel (Amer *et al.* 2001, Miura *et al.* 2006). In this study, analyzing samples collected from animals during the early spermatogenic period, we observed that DHP plasma levels increased significantly first in fish, where the testes contained type B spermatogonia, compared with testes containing type A spermatogonia. Interestingly, this increase occurred in samples collected in March, but not in February, indicating that the transition from early type B to late type B spermatogonia that we have recorded histologically during this period is associated with the increased DHP plasma levels. Since our *in situ* hybridization localized *pgr* mRNA expression to Sertoli cells, we can assume a Sertoli cell-mediated effect of DHP on germ cell development, a situation that also characterizes androgen effects on spermatogenesis. Hence, it is possible that DHP, via a Pgr-mediated pathway, participated in stimulating the transition to the rapid mode of proliferation typical for the late type B spermatogonia.

In male salmonid fish, plasma DHP levels increased with the appearance of meiotic cells in the testis of Japanese huchen, *Hucho perryi* (Amer *et al.* 2001), and rainbow trout, *Oncorhynchus mykiss* (Scott & Sumpter 1989). This is in agreement with this study, where we found a significant increase in plasma DHP levels from stage SG-B to stage SC in Atlantic salmon, suggesting that DHP may be functionally related to the initiation of meiosis as well, as had been demonstrated

experimentally for Japanese eel (Miura *et al.* 2006). However, considering our *in situ* hybridization results that Sertoli cells contacting larger clones with further differentiated germ cells were less intensely/not stained, it seems that once DHP participated in stimulating the further development of early type B spermatogonia, *pgr* mRNA expression may become down-regulated in the Sertoli cells now contacting late type B spermatogonia. Still, without data on the half-life time of Pgr protein, we cannot exclude that Pgr protein persists longer than its mRNA. Taken together, the present data are not conclusive as regards a potential function of DHP/Pgr in meiosis, and other experimental approaches will be required to address this question.

A possible explanation for the observed increase in plasma DHP levels that were associated with the transition from early to late type B spermatogonia and again to spermatocytes is an observation made in rainbow trout, where the enzyme 20 β -hydroxysteroid dehydrogenase, converting 17 α (OH)P₄ to DHP, was localized to spermatogonia (Vizziano *et al.* 1996). Hence, the increasing number of spermatogonia typically observed at the start of testis growth might pave the way, by providing ligand for Pgr present in Sertoli cells contacting early spermatogonia, for stimulating germ cell development toward meiosis. Down-regulating *pgr* mRNA expression in Sertoli cells that envelope more advanced germ cell stages and cessation of the production of new spermatogonial cysts may be the two aspects contributing to terminate the DHP/Pgr-driven regulatory loop that we postulate to operate during the rapid spermatogonial proliferation phase in Atlantic salmon.

In summary, we have cloned a single cDNA coding for a Pgr in the Atlantic salmon; there are no indications suggesting that another *pgr* gene is present in the salmon genome. Pharmacological characterization, cellular localization, and quantification of *pgr* mRNA in salmon testis in relation to other reproductive parameters suggest that the salmon Pgr, which is expressed in Sertoli cells and best activated by its natural ligand (DHP), may be involved in the regulation of early spermatogenesis, particularly the proliferation of type B spermatogonia. Additional studies using models allowing more direct experimental approaches, e.g. testis tissue culture studies, are needed to obtain further information on the roles of DHP/Pgr in the regulation of spermatogenesis.

Materials and Methods

Maintenance, photoperiod treatment, and sampling of fish

Previously immature, two sea-winter old salmon had been kept in sea cages at the Institute of Marine Research (Matre, Norway; 61°N) under NL conditions for 19 months until the start of the trial. An initial control sample was collected on 8 January. Starting on 1 February, half of the animals were exposed to

additional LL, while the other half remained under NL conditions. Samples were then collected at four time points: 18 February, 19 March, 25 April, and 11 June. In June, maturation was clearly visible in all males of the NL group, and the experiment was terminated.

For tissue and blood sampling, the fish were netted from the sea cages, immediately anesthetized with 6 ppt metomidate (Syndel, Victoria, BC, Canada), and weighed (total body weight); blood was collected in heparinized syringes from the caudal veins, and gonads were excised and weighed. The GSI was calculated as: GSI (%) = gonad weight (g) \times 100/total body weight (g). Testis tissue samples were either shock frozen in liquid nitrogen and then stored at -80°C for gene expression analysis or fixed for different purposes.

Testis histology and sex steroid quantification

For routine histological analysis, a testis tissue fragment was fixed in phosphate buffered 4% m/v paraformaldehyde and 2% v/v acetic acid, dehydrated, and embedded in paraffin wax, according to conventional techniques; 5 μm sections were stained with hematoxylin and eosin.

Stages of spermatogenesis were determined by identifying the most advanced germ cell generation, using criteria and a terminology that can be applied to male germ cells in all vertebrates (Schulz *et al.* 2010). The animals were assigned to one of the three following stages of spermatogenesis. In stage SG-A, the testes contained only type A spermatogonia that showed a large nucleus ($\sim 10\ \mu\text{m}$; Supplementary Figure 1A, see section on supplementary data given at the end of this article) with little heterochromatin and one or two prominent nucleoli. This largest type of spermatogonia often occurred as single cells in contact with one or two Sertoli cells. In stage SG-B, type B spermatogonia were present in addition to type A spermatogonia. Early generations of type B spermatogonia were present in pairs or small groups and had a smaller nucleus ($\sim 7\ \mu\text{m}$; Supplementary Figure 1B) that stained more intensely with hematoxylin. Late type B spermatogonia occurred in larger groups and showed an increased amount of heterochromatin speckles distributed over, in general, more darkly stained nucleus (Supplementary Figure 1C), and the cytoplasmic area stained more intensely with eosin than in the previous germ cell generations. In stage SC, spermatocytes were present, which occurred in large groups and were characterized by the presence of darkly staining meiotic chromosomes at different stages of condensation during the first meiotic prophase (Supplementary Figure 1D). In two out of the 12 animals found in stage SC, spermatids were found as well (Supplementary Figure 1D).

Aliquots of blood plasma were obtained by centrifugation at 3000 *g* at 4°C for 10 min and stored at -80°C until analyzed for DHP. Since low levels of DHP were expected, 500 μl plasma sample were extracted with 5 ml diethyl ether, and the ether was decanted. This procedure was repeated twice, and the ether fractions were combined and evaporated to dryness. The residue was dissolved in 5 ml of 70% (v/v) methanol and stored at -20°C overnight for defatting. After centrifugation at 3000 *g* at 4°C for 10 min, the methanol–water supernatant was decanted, evaporated to dryness, and the dry residue was

dissolved in 125 µl RIA buffer and assayed as described previously (Schulz *et al.* 1994). In some cases, the available volume of plasma was not sufficient, so that the number of samples analyzed for DHP plasma levels was smaller than for *pgr* mRNA expression.

Cloning and sequence analysis of salmon *pgr* cDNA

Total RNA was extracted from adult salmon ovary using the FastRNA Pro Green kit (MP Biomedicals, Solon, OH, USA). Poly(A)-rich salmon ovary RNA was isolated using Dynabeads-oligo dT₂₅ (DynaL A.S., Oslo, Norway) and reverse transcribed to 5'- and 3'-RACE ready cDNA using a SMART RACE cDNA amplification kit (Clontech) following the manufacturer's instructions.

To obtain a partial salmon *pgr* cDNA sequence, 2 µl random hexamer-primed salmon ovary cDNA were used as template in a PCR with primer set 2699 and 2700 (Supplementary Table 1, see section on supplementary data given at the end of this article), corresponding to highly conserved amino acid sequences found in known PGRs. The PCR was carried out in a 50 µl volume using the Advantage 2 PCR system (Clontech) in a Perkin-Elmer 2400 Thermal Cycler (Applied Biosystems, Foster City, CA, USA) under the following cycling conditions: denaturation at 94 °C for 10 s, followed by 35 cycles of 94 °C for 10 s, 55 °C for 10 s, 68 °C for 1 min. DNA fragments were subcloned into pcDNA3.1/V5-His-TOPO vector (Invitrogen), and plasmid DNA of two clones was prepared for DNA sequence analysis.

To isolate the 5'- and 3'-ends of the salmon *pgr* cDNA, gene-specific primers (2722 and 2720; Supplementary Table 1) based on the consensus nucleotide sequence of the above-mentioned two clones were used in combination with a universal primer mix (UPM) for 5'- and 3'-RACE respectively. These initial 5'- and 3'-RACE products were then used for nested PCR amplifications using gene-specific nested primers (2721 and 2723; Supplementary Table 1) respectively, in combination with a nested universal primer (NUP). To obtain more sequence information at the 3'-end of the salmon *pgr*, an additional 3'-RACE was performed using the gene-specific primers 2767 and 2768 (Supplementary Table 1) in combination with UPM and NUP respectively. Both the UPM and NUP were supplied with the SMART RACE cDNA amplification kit (Clontech). All RACE reactions were carried out according to the manufacturer's instructions in a Perkin-Elmer 2400 Thermal Cycler (Applied Biosystems) using Advantage 2 polymerase (Clontech). RACE products were subcloned into pcDNA3.1/V5-His-TOPO vector (Invitrogen).

The ORF of the salmon *pgr* was PCR amplified using primers 2794 and 2796 (Supplementary Table 1), subcloned into pcDNA3.1/V5-His-TOPO vector, and checked for the correct orientation by DNA sequence analysis. DNA sequence analyses were performed using dye terminator cycle sequencing chemistry (Applied Biosystems).

Phylogenetic analysis

After obtaining the salmon *pgr* cDNA sequence, a BLAST homology search was performed. The alignment of multiple

nuclear PGR sequences were performed using the MegAlign program of the Lasergene software package (DNASTAR, Inc., Madison, WI, USA) with the Clustal V (PAM 250) algorithm, and percentage identities were calculated. We only selected (deduced) PGR amino acid sequences from studies that experimentally demonstrated progesterones binding to the receptors (see Supplementary Table 2 for the respective GenBank accession numbers). The phylogenetic tree was constructed using the neighbor-joining method with a bootstrap value of 1000 trials for each position and rooted by two types of Atlantic salmon androgen receptor (data not shown).

Transactivation assays for salmon *Pgr*

Receptor activation was measured using a reporter gene assay as described previously (Chen *et al.* 2010). Briefly, HEK 293T cells were seeded in 10 cm dishes in DMEM supplemented with 10% v/v fetal bovine serum (FBS), glutamine, and penicillin/streptomycin (Gibco) at 37 °C in a 5% CO₂ incubator. After 24 h, the cells were co-transfected using a standard calcium phosphate precipitation method with 1 µg of the salmon *pgr* expression plasmid and 7 µg of pGL3-MMTV-Luc plasmid. After 5–6 h, the transfected cells were transferred to 24-well plates. The next day, the medium was replaced by transactivation assay medium (DMEM without phenol red, supplemented with 0.2% v/v charcoal-stripped FBS, glutamine, and non-essential amino acids) containing different steroids (in duplicate) with final concentrations ranging between 0.1 nM and 10 µM. After incubation at 37 °C for 24–36 h, the cells were harvested in lysis mix and stored at –80 °C. Luminescence was measured in a Packard TopCount NXT luminometer (Perkin Elmer Life Sciences, Meriden, CT, USA). Each compound was tested in three independent experiments using cells from different transfections.

The following steroids were used in this study: DHP, 20β-S, P₄, 17α(OH)P₄, testosterone, 11-KT, E₂, cortisol, and the synthetic progestin promegestone (R5020). All steroids were purchased from Sigma–Aldrich (Zwijndrecht, The Netherlands).

Localization and quantification of *pgr* expression

Localization of receptor expression by *in situ* hybridization was done as described previously (Chen *et al.* 2010), using 10 µm cryosections prepared from paraformaldehyde-fixed testis tissue from fish sampled during the onset of spermatogenesis. Cell nuclei were visualized with DAPI counterstaining (Vectashield with DAPI; Vector Laboratories, Burlingame, CA, USA). Specific primers (3168 and 3198; Supplementary Table 1) were designed to amplify a salmon *pgr* cDNA fragment for sense and antisense digoxigenin-labeled cRNA probe synthesis.

To assess the tissue specificity of *pgr* expression in Atlantic salmon, brain, gill, head kidney (including interrenal cells), heart, intestine, kidney, liver, spleen, muscle, ovary, pituitary, and stomach tissue samples were collected from three previtellogenic females in January, and testis tissue samples were collected from three immature fish in January, and used

for RNA isolation and cDNA synthesis, as described previously (Andersson *et al.* 2009).

Primers 2831 and 2832 (Supplementary Table 1) were designed to detect salmon *pgr* using quantitative real-time PCR (qPCR). The specificity and efficiency of this *pgr* primer set were tested with qPCR on serial dilutions of salmon testis cDNA as described elsewhere (Vischer *et al.* 2003). Atlantic salmon *ef1 α* was used as reference gene as described in detail previously (Andersson *et al.* 2009); no significant differences in expression levels were found in the testis samples analyzed (Supplementary Figure 5). All qPCR were performed in 20 μ l volume, and C_t values were determined in a 7900HT Real-Time PCR System (Applied Biosystems) using default settings. To calculate gene expression data, the $\Delta\Delta C_t$ method was used, as described in detail previously (Bogerd *et al.* 2001).

The expression levels in the different salmon tissues were normalized to *ef1 α* mRNA levels and are shown relative to the tissue showing the highest level of expression (immature testis tissue), which was set to 1. Expression levels in testis samples were calculated in two ways: normalized to *ef1 α* mRNA only (see Fig. 4E and F) and normalized to *ef1 α* mRNA and corrected according to Kusakabe *et al.* (2006) see Fig. 4G and H: pgr expression levels = (qPCR value/*ef1 α* value) \times (total RNA amount extracted/tissue weight for RNA extraction) \times (testis weight/body weight). The results are shown relative to immature testis tissue (i.e. stage SG-A), which was set to 1.

Statistical analysis

Analyses of GSI, DHP plasma levels, and *pgr* expression data were carried out on groups that were defined according to the histological analysis of testis development (stages SG-A, SG-B early or late, and SC) and exposure to NL or LL. Data were subjected to ANOVA, followed by a Tukey's unequal N HSD test to identify differences among groups. Differences between testes in the SG-A and SG-B stages, collected in a given month, were analyzed using a Student's *t*-test. In all case, significance was accepted at $P < 0.05$. Data are presented as the mean \pm S.E.M.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-10-0224>.

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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