Function of progestin and its nuclear receptor in fish spermatogenesis

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Function of progestin and its nuclear receptor in fish spermatogenesis

Functie van progesteron en haar nucleaire receptor in vis spermatogenese

(met een samenvatting in het Nederlands)

Proefschrift

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Table of Contents

Chapter 1

General Introduction

General structure of the testis in vertebrates

The propagation of animal species depends on the development of reproductive organs that support the differentiation of the germ cell lineage into two types of functional gametes: sperm and eggs. This thesis will concentrate on the male gender.

The morphology of the adult testis is remarkably similar among vertebrates. It is composed of two distinct compartments, the intertubular (or interstitial) and the tubular compartment (Grier 1981). The intertubular compartment consists of the interstitial tissue, containing blood and lymphatic vessels, and Leydig cells which mainly secrete sex steroid hormones, including androgens, macrophages and mast cells, neural and connective tissue cells, the latter being continuous with the tunica albuginea (Koulish *et al*. 2002), i.e. the testis organ wall. The tubular compartment contains the germinal epithelium defined as a tripartite structure consisting of an acellular basement membrane, Sertoli cells and germ cells, which are found at different stages of development (Grier 1993). In the testis, germ cells are associated with a somatic cell type, the Sertoli cells, and this association forms the germinal epithelium, an evolutionary conserved feature among all vertebrates (Pudney 1993). Importantly, germ cells can only survive in close and continuous interaction with Sertoli cells. From a regulatory point of view, it is moreover crucial to realize that Sertoli cells, but not germ cells, are the target (i.e. express the receptors) for the reproductive hormones regulating spermatogenesis in vertebrates (Matta *et al*. 2002).

The general testis structure among vertebrates allows distinguishing two patterns, one present in amniotes (reptiles, birds, mammals), the other in anamniotes (fish, amphibia) (Pudney 1995).

In amniote vertebrates, the germinal compartment is found in – except for birds – unbranched seminiferous tubules (Fig 1A). The seminiferous tubules contain only two cell types: germ cells in different stages of spermatogenesis, and Sertoli cells. The immature germ cells, the spermatogonia, are located at the periphery of the seminiferous epithelium and are in contact with the basement membrane. Spermatocytes and spermatids form consecutive layers along the lateral surface of Sertoli cells towards the lumen of the tubules. Mature, elongated spermatids border the lumen of the tubules into which they are released during spermiation. Therefore, there is a stratification of germ cells at different phases of maturation within the seminiferous tubules of amniotes. This setting has also been defined as postspermatocystic (Grier 1993), a description that becomes clear in comparison with anamniote vertebrates (see below).

In the tubules of anamniote vertebrates, the functional unit of spermatogenesis is the spermatogenic cyst, usually referred to as cyst (Callard 1996). Cysts are formed when a single primary, or undifferentiated type A, spermatogonium becomes enveloped by a Sertoli cell. These cells then undergo a species-specific number of mitotic divisions, thereby producing differentiating type A and then type B spermatogonia. The cyst is now composed of numerous germ cells surrounded by the cytoplasmic extensions of Sertoli cells, which form the wall of the cyst. During the mitotic expansion of the spermatogonia,

also the Sertoli cells increase in number by mitosis (Schulz *et al*. 2005; Leal *et al*. 2009). Following meiosis and spermiogenesis, germ cell maturation then finishes with the formation of spermatozoa, which are released, or spermiated, into the tubular lumen by rupturing of the cyst wall.

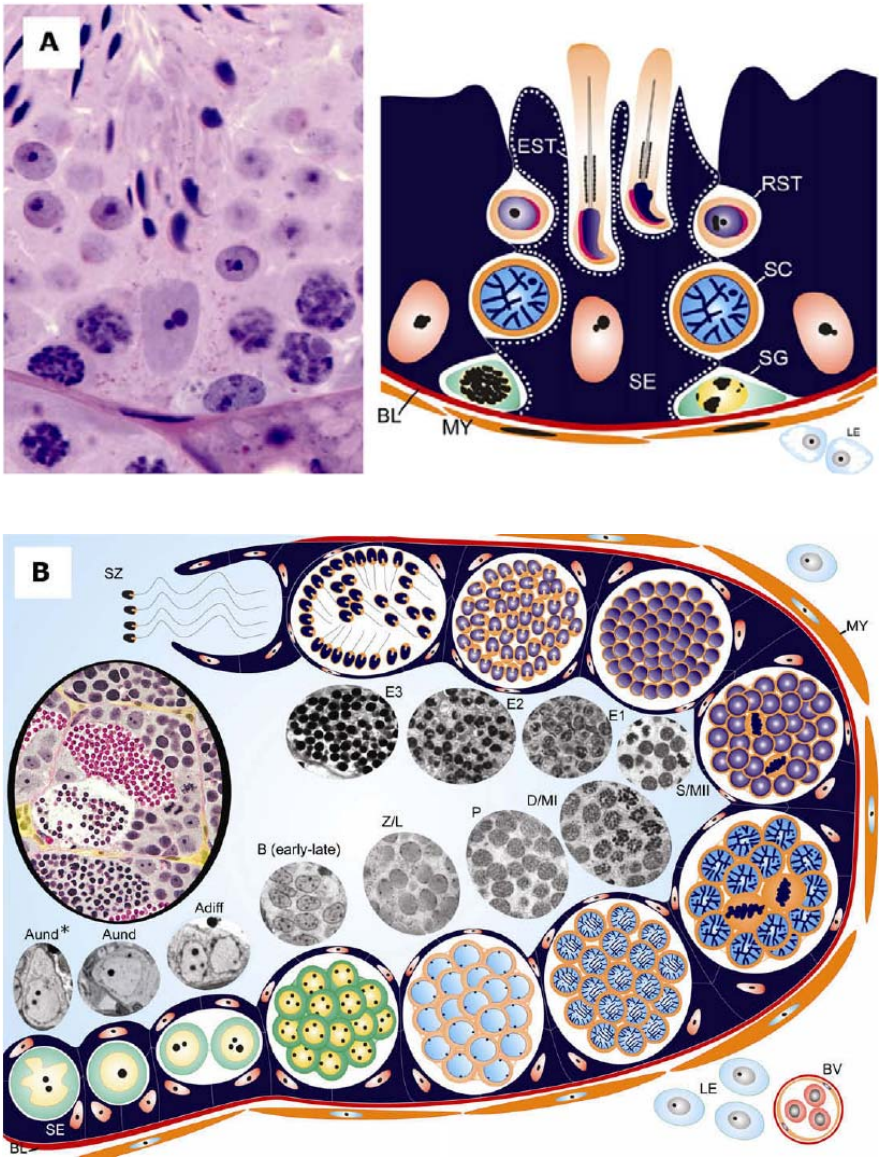

Fig. 1. Comparison of mammalian (A, mouse) and fish (B, zebrafish) testes. Segments of spermatogenic tubules are shown to illustrate the differences in Sertoli/germ cell relation between cystic (B) and non-cystic (A) spermatogenesis. The germinal epithelium contains Sertoli (SE) and germ cells, delineated by a basal lamina (BL) and peritubular myoid cells (MY). The interstitial Leydig cells (LE) and blood vessels (BV) are shown. A. Also shown are: spermatogonium (SG), spermatocyte (SC), round spermatid (RST), and elongated spermatid (EST). B. Shown are: type A undifferentiated* spermatogonium (Aund*), type A undifferentiated spermatogonium (Aund), type A differentiated spermatogonia (Adiff), spermatogonia type B [B (early-late)], leptotenic/zygotenic primary spermatocytes (L/Z), pachytenic primary spermatocytes (P), diplotenic spermatocytes/metaphase I (D/MI), secondary spermatocytes/metaphase II (S/MII), early (E1), intermediate (E2) and final spermatids (E3), and spermatozoa (SZ). This figure is adapted from Schulz *et al*. 2010.

Hence, the typical histological picture of fish testis is a tubular compartment containing spermatogenic cysts, which are formed by a dynamic group of Sertoli cells surrounding and nursing one synchronously developing germ cell clone. Cysts of different sizes contain groups of germ cells in different stages of spermatogenesis (Fig 1B).

This organization of the testis makes certain anamniotes suitable models for investigating germ cell development and maturation. For instance, the synchronized development of germ cell clones in association with their specific group of Sertoli cells provides the opportunity to study selected germ cell stages for Sertoli/germ cell interactions. Moreover, since a given Sertoli cell usually is in contact with a single germ cell clone only, and hence a single stage of spermatogenesis, the Sertoli/germ cell relation in anamniotes is much less complex than in the amniote testis. Thus, due either to the anatomical organization of the testis, or to the structural simplicity of the germinal units (i.e. Sertoli cells are in contact with one type of germ cell only at a time), fish, the most numerous group of species among vertebrates, can provide excellent experimental animal models for investigating many basic questions of male reproduction.

Spermatogenesis in fish

Spermatogenesis is a cyclic, complex, highly organized and tightly coordinated process, in which single, diploid spermatogonial stem cells can go through two types of division; either a division for self-renewal that guarantees long-term fertility, or a division that provides two daughter cells, which develop – after a number of mitotic divisions – into spermatocytes that complete meiosis to produce haploid spermatids. These cells enter spermiogenesis, i.e. undergo a series of morphological transformations to differentiate into spermatozoa. These general characteristics of spermatogenesis are fairly conserved throughout vertebrates (and also invertebrates).

The process can morpho-functionally be divided in three different phases: the mitotic or spermatogonial phase with the different generations of spermatogonia (*i.e.*, undifferentiated spermatogonia including the stem cells, and differentiated or differentiating spermatogonia); the meiotic phase with the primary and secondary spermatocytes; and the spermiogenic phase with the haploid spermatids emerging from meiosis and differentiating - without further proliferation - into motile, flagellated genome vectors, the spermatozoa. Except for the mitotic phase, which provides for the exponential expansion of germ cell numbers and which is subject to different regulatory systems in different species, the developmental events during the other two phases seem quite similar and highly conserved among vertebrates.

During the mitotic phase, the number of spermatogonial generations can vary among fish species (Ando *et al*. 2000). Therefore, the use of the same nomenclature for the same cell types is required. Recently, an attempt was made to classify fish germ cells using the same terminology as for higher vertebrates (Schulz *et al*. 2010). Based on their morphology, the spermatogonial cells can be classified in two types: type A and type B.

Functionally, type A spermatogonial are divided in undifferentiated (A_{und}), comprising also the spermatogonial stem cells (SSC), and differentiated spermatogonia (A_{diff}). A_{und} give rise to A_{diff} that share some morphological characteristics with $A_{und.}$ Irreversible commitment to further maturation and a change in a number of morphological aspects gives rise to the rapidly dividing type B spermatogonia, which can occur in a number of generations. In species showing several spermatogonial generations, a differentiation can be made based on cell/nuclear size and number of cells per cysts into early and late type B spermatogonia (B_{early} and B_{late}, respectively).

Endocrine control of spermatogenesis by hormones

Spermatogenesis is under endocrine control of the pituitary glycoprotein hormones, FSH and LH, and the sex steroids hormones produced in Leydig cells. Moreover, locally produced growth factors participate in the regulation of germ cell development.

In mammals, the exclusive target of FSH in the testis is the Sertoli cell; no other testicular cells, including germ cells, express the FSH receptor. One aspect of FSH-regulated Sertoli cell function is the production of growth factors that modulate germ cell proliferation or have endocrine feedback effects (Plant and Marshall 2001). The primary target cell type of LH is the Leydig cell, which produces testosterone (Dufau 1998). Testosterone feeds back to the brain and pituitary, thereby inhibiting LH and FSH release in a classic negative feedback loop (Fink 1979). Testosterone is moreover essential for supporting spermatogenesis directly at the testicular level. Again, Sertoli cells come into play, since they express, in contrast to germ cells, the androgen receptor (AR). Sertoli cell AR expression, finally, is required in mammals for all stages of spermatogenesis beyond the first meiotic prophase (Petersen and Soder 2006).

Endocrine regulation of piscine spermatogenesis has been investigated mainly in prepubertal individuals, while information on the mechanisms and hormone-dependent phases of adult piscine spermatogenesis is scarce. In contrast to mammals, in which the specific activities of both gonadotropins are clearly defined, given the highly specific interactions between each hormone and its receptor, Fshr and Lhcgr, respectively, the binding of hormones by the Fshr and Lhcgr in teleost fish seemed to be less specific. Studies using hormones from the same (or very closely related) species (African catfish, *Clarias gariepinus*: Jan *et al*. 2001, García-López *et al*. 2009; Channel catfish, *Ictalurus punctatus*: Sampath Kumar *et al*. 2000; Japanese eel, *Anguilla japonica*: Kazeto *et al.* 2008; zebrafish, *Danio rerio*: So *et al*. 2005; but with the possible exception of rainbow trout, *Salmo gairdneri*: Sambroni *et al.* 2007), showed that the Fshr has a preference for FSH, but was also (partially) activated by LH, so that high LH concentrations, such as during spawning, might activate Fshr-dependent signaling. However, recent experimental data in zebrafish did not provide evidence for LH-mediated activation of Fshr-dependent processes in adult males *in vivo* (García-López *et al*. 2010). Functionally, both FSH and LH are strong steroidogenic hormones in fish. Recently, Fshr protein (Japanese eel) (Ohta *et al*. 2007) and mRNA (African catfish, García-López *et al*. 2009; zebrafish,

García-López *et al*. 2010) have been demonstrated in teleost Leydig cells. Hence, also in fish, Sertoli cell functions are predominantly regulated by FSH, while Leydig cell steroidogenesis is regulated by both, LH and FSH.

Besides FSH and LH, sex steroids, such as progestagens, androgens, and estrogens have important and distinct roles in controlling fish spermatogenesis. Among them, androgenic sex steroids are considered being the most important hormones regulating spermatogenesis. In fish, beside testosterone, the quantitatively dominating steroids synthesized in the testis are 11-oxygenated androgens, such as 11-ketotestosterone (11-KT; Borg 1994). Androgens are effective in supporting either the whole process of spermatogenesis (Miura *et al*. 1991a; Almeida *et al*. 2009), or at least some steps such as initiation of spermatogenesis *in vivo* (Cavaco *et al*. 1998; de Waal *et al.* 2009) and *in vitro* (Miura *et al*. 1991b), or spermatocyte formation (Fostier *et al*. 1982; Campbell *et al*. 2003).

Estrogens generally are considered 'female' hormones, but 17*β*-estradiol (E_2), a natural estrogen in vertebrates, is found in blood serum also of male teleosts, although at rather low concentrations (Miura *et al.* 1999; Amer *et al.* 2001). On the other hand, in the Japanese eel, already 10 pg E_2 per ml was sufficient to induce spermatogonial stem cell renewal (Miura *et al*. 1999). A similar effect was found in medaka, while high doses had inhibitory effects (Song and Gutzeit, 2003). Inhibitory effects of higher doses of estrogen on spermatogenesis have also been described in adult male zebrafish (de Waal *et al*. 2009), probably reflecting a negative feedback-mediated down-regulation of androgen production. These findings clearly indicate that estrogen is also relevant for regulating spermatogenesis.

The major progestin in teleosts is not progesterone, as in tetrapods, but 17,20*β*-dihydroxypregn-4-en-3-one (DHP) or, in certain species, 17,20*β*,21-trihydroxy-pregn-4-en-3-one (20*β*-S). In male fish, DHP or 20*β*-S are present in the blood at high levels during the spermiation period, and DHP has been reported to stimulate sperm hydration (Ueda *et al*. 1985) and acquisition of sperm motility (Miura *et al*. 1991a, 1992). During the reproductive cycle of salmonid fish, there is another small peak of DHP blood plasma level during the progression of spermatogonial proliferation (Dépêche and Sire, 1982; Scott and Sumpter, 1989; Vizziano *et al*. 1996a). More recently, it was found that DHP induces the entry of male germ cells into meiosis (Miura *et al*. 2006), involving the DHP-mediated expression of trypsinogen by Sertoli cells (Miura *et al*. 2009), but it is not clear yet what might be the possible function of the early peak in plasma DHP levels. Taken together, these data indicate that also progestagenic sex steroids have important functions in regulating spermatogenesis.

Mechanism of hormone action

All hormones bind to target cell receptors. Receptor activation then initiates specific responses that collectively are described as mechanism of hormone action.

It has been known for a long time in both mammals and fish that the cell

membrane-embedded receptors for FSH and LH belong to the G protein-coupled receptor (GPCR) family and activate the enzym adenylate cyclase via G protein $G\alpha_s$, which in turn leads to increased levels of intracellular cAMP (Oba *et al.* 1999a, b). The increase of intracellular cAMP activates protein kinase A (PKA), and the catalytic subunit of PKA in turn phosphorylates structural proteins, enzymes and transcriptional regulators, such as cAMP responsive element (CRE)-binding proteins (CREBs) will lead to activation of CRE on the promoter of target genes and ultimately mRNA synthesis of primary response genes of gonadotropin action (Mukherjee *et al.* 1996). Although cAMP is generally accepted to be the principal second messenger mediating FSH and LH actions, evidence also suggests that other second messengers may have a role in their signaling, such as activation of phospholipase C (PLC). After formation of the gonadotropin-receptor complex, activated PLC will increase inositol-1,4,5-triphosphate (IP3) and diacylglycerol (DAG) production, both of which can act as secondary messengers for mobilization of calcium and activation of protein kinase C (PKC), respectively (Leung and Steele 1992).

Steroid hormone receptors are found within target cells, either in the cytoplasm or in the nucleus. The ultimate destination of steroid hormone-receptor complexes is the nucleus, where the complex acts as a transcription factor, binding to DNA and activating or repressing one or more genes. Activated genes create new mRNA that directs the synthesis of new proteins. Any hormone that alters gene expression is said to have a genomic effect on the target cell (Beato 1989). When steroid hormones activate genes to direct the production of new proteins, there is usually a lag time between hormone-receptor binding and the first measurable biological effects. This lag time can be as long as 90 minutes. Consequently, steroid hormones used to be considered to not being involved in triggering rapid responses. In recent years, however, it has been discovered that some steroid hormones, including estrogen, aldosterone and progesterone, also have cell membrane-associated receptors linked to rapid signal transduction pathways. These receptors enable those steroid hormones to initiate rapid, non-genomic responses in addition to their slower genomic effects (Lösel and Wehling 2003).

Aim and outline of this thesis

The principal hormones controlling vertebrate spermatogenesis are the pituitary gonadotropins, FSH and LH, and androgens. The present knowledge in fish indicates that the main function of FSH during early stages of spermatogenesis is stimulating the production of spermatogenesis-inducing steroids, such as 11-ketotestosterone, the major androgen of teleost fish (Ohta *et al*. 2007). However, as mentioned above, also other sex steroid hormones, like DHP, play important roles during different stages of spermatogenesis. Some information has been published as regards more advanced stages of spermatogenesis, such as entry into meiosis, sperm hydration and sperm motility (see above), but there is little information on the mitotic stage of spermatogenesis, although in salmonid fish, there is a small peak of DHP blood plasma levels during the progression of spermatogonial proliferation (Scott and Sumpter, 1989; Amer *et al*. 2001). Moreover,

studies on testicular steroid metabolism in rainbow trout showed that conversion of 17α-hydroxy-4-pregnen-3-one (17α(OH)P4) to DHP was highly efficient in immature testis tissue that contained spermatogonia only (Dépêche and Sire 1982), and later work showed that DHP production may reside in germ cells (Vizziano *et al*. 1996b). Taken together, these data open the possibility that the DHP may be involved in the regulation of early (mitotic) stages of spermatogenesis in teleost fish.

The biological activity of progesterone is mediated via specific receptors. The classic nuclear progesterone receptor (Pgr) functions primarily as ligand-dependent transcription factor to regulate target gene expression (Conneely *et al.* 2002), while membrane-associated progesterone receptors are involved in non-genomic mechanisms (Thomas 2008). The physiological significance of membrane-associated progesterone receptors is still a matter of discussion (Krietsch *et al*. 2006). This thesis concentrates its efforts on understanding the Pgr-mediated effect of DHP on early spermatogenesis.

Zebrafish (*Danio rerio,* Cyprinidae) is a vertebrate model system offering the attractive combination of being simple to maintain and suitable for studies on development, genetics, diseases, and physiology (Briggs 2002). Zebrafish are also used for basic studies on the biology of reproduction (Leal *et al.* 2009; Ge 2005). To develop our understanding of the two main testicular functions, spermatogenesis and steroidogenesis, and to elucidate the possible Pgr role(s) in this context, the studies described in **Chapter 2** were aimed at cloning the zebrafish nuclear progesterone receptor (*pgr*) cDNA, establishing the pharmacological characterization of the zebrafish Pgr, as well as *pgr* mRNA expression patterns during ontogenesis, and in different adult tissues. We also studied the capacity of zebrafish testicular explants to produce DHP under gonadotropin stimulation and the ability of DHP to modulate androgen release in a Pgr-dependent manner.

To further understand the function of DHP during early stages of spermatogenesis in zebrafish, we have used an *in vivo* experimental model that interrupted adult spermatogenesis and resulted in testis tissue enriched in type A spermatogonia (de Waal *et al*. 2009). Testis tissue from these males was then used in **Chapter 3** to study effects of DHP on the proliferation and differentiation behaviour of spermatogonia *in vivo* and *ex vivo*, the latter based on a recently developed, primary zebrafish testis tissue culture system (Leal *et al*. 2009). Moreover, we determined the expression of genes reflecting aspects of Sertoli cell function and spermatogenic development in adult zebrafish testis after DHP treatment, in order to identify potential factors mediating DHP action.

Subsequently, it was intended to broaden the data basis as regards the role of Pgr in fish spermatogenesis from literature data in eel (Anguilliformes) and the present studies on zebrafish (Cypriniformes), to other, unrelated orders, the Salmoniformes and Gadiformes. Several economically relevant species (for aquaculture biotechnology) belong to these orders, and the precocious activation of spermatogenesis is a significant problem compromising the sustainability of salmonid and gadoid aquaculture. The two species chosen (Atlantic salmon – *Salmo salar*; Atlantic cod – *Gadus morhua*) are both showing modes of spermatogenesis that differ from the ones shown by eel and zebrafish,

respectively. Therefore, we report the pharmacological characterization of the salmon Pgr and cod Pgr in **Chapters 4 and 5**, respectively. Moreover, we determine the cellular localization of the *pgr* mRNA in testis by *in situ* hybridization, and we analyzed changes in testicular *pgr* mRNA levels during early spermatogenesis in fish.

In **Chapter 6** the findings described in this thesis are summarized and discussed.

References

Almeida FF, Taranger GL, Norberg B, Karlsen O, Bogerd J, Schulz RW 2009 Photoperiod-modulated testis maturation in Atlantic cod (*Gadus morhua*, L.). *Biology of Reproduction* **80** 631-640.

Amer MA, Miura T, Miura C & Yamauchi K 2001 Involvement of sex steroid hormones in the early stages of spermatogenesis in Japanese huchen (*Hucho perryi*). *Biology of Reproduction* **65** 1057-1066.

Ando N, Miura T, Nader MR, Miura C, Yamauchi K 2000 A method for estimating the number of mitotic divisions in fish testes. *Fisheries Science* **66** 299-303.

Beato M 1989 Gene regulation by steroid hormones. *Cell* **56** 335-344.

Bogerd J, Blomenröhr M, Andersson E, van der Putten HH, Tensen CP, Vischer HF, Granneman JC, Janssen-Dommerholt C, Goos HJ, Schulz RW 2001 Discrepancy between molecular structure and ligand selectivity of a testicular follicle-stimulating hormone receptor of the African catfish (*Clarias gariepinus*). *Biology of Reproduction* **64** 1633-1643.

Borg B 1994 Androgens in teleost fishes. *Comparative Biochemisty and Physiology Part C* **109** 219-245.

Briggs JP 2002 The zebrafish: a new model organism for integrative physiology. *American journal of physiology. Regulatory, integrative and comparative physiology* **282** R3-R9.

Callard GV 1996 Endocrinology of Leydig cells in nonmammalian vertebrates. In: Payne AH, Hardy MP, Russell LD (Eds.), *The Leydig Cell*. pp. 308-331. Cache River Press, Vienna, IL, USA.

Campbell B, Dickey JT, Swanson P 2003 Endocrine changes during onset of puberty in male spring Chinook salmon, *Oncorhynchus tshawytscha*. *Biology of Reproduction* **69** 2109-2117.

Cavaco JEB, Vilrokx C, Trudeau VL, Schulz RW, Goos HJT 1998 Sex steroids and the initiation of puberty in male African catfish, *Clarias gariepinus. American Journal of Physiology* **44** R1793-R1802.

Conneely OM, Mulac-Jericevic B, DeMayo F, Lydon JP, O'Malley BW 2002 Reproductive functions of progesterone receptors. *Recent Progress in Hormone Research* **57** 339-355.

de Waal PP, Leal MC, García-López A, Liarte S, de Jonge H, Hinfray N, Brion F, Schulz RW, Bogerd J 2009 Oestrogen-induced androgen insufficiency results in a reduction of proliferation and differentiation of spermatogonia in the zebrafish testis.

Journal of Endocrinology **202** 287-297.

Dufau ML 1998 The luteinizing hormone receptor. *Annual Review of Physiology* **60** 461-496.

Dépêche J, Sire O 1982 In vitro metabolism of progesterone and 17*α*-hydroxyprogesterone in the testis of the rainbow trout, *Salmo gairdneri* Rich., at different stages of spermatogenesis. *Reproduction, Nutrition, development* **22** 427-438.

Fink G 1979 Feedback actions of target hormones on hypothalamus and pituitary with special reference to gonadal steroids. *Annual Review of Physiology* **41** 571-585.

Fostier A, Billard R, Breton B, Legendre M, Marlot S 1982 Plasma 11-oxotestosterone and gonadotropin during the beginning of spermiation in rainbow trout (*Salmo gairdneri* R.). *General and Comparative Endocrinology* **46** 428-434.

García-López A, Bogerd J, Granneman JC, van Dijk W, Trant JM, Taranger GL, Schulz RW 2009 Leydig cells express follicle-stimulating hormone receptors in African catfish. *Endocrinology* **150** 357-365.

García-López A, de Jonge H, Nóbrega RH, de Waal PP, van Dijk W, Hemrika W, Taranger GL, Bogerd J, Schulz RW 2010 Studies in zebrafish reveal unusual cellular expression patterns of gonadotropin receptor messenger ribonucleic acids in the testis and unexpected functional differentiation of the gonadotropins. *Endocrinology* **151** 2349-2360.

Ge W 2005 Intrafollicular paracrine communication in the zebrafish ovary. The state of the art of an emerging model for the study of vertebrate folliculogenesis. *Molecular and Cellular Endocrinology* **237** 1-10.

Grier HJ 1981 Cellular organization of the testis and spermatogenesis in fishes. *American Zoologist* **21** 345-357.

Grier HJ 1993 Comparative organization of Sertoli cells including the Sertoli cell barrier. *In* Russell LD and Griswold ND (eds.), *The Sertoli cell,* pp. 704-730. Cache River Press, Clearwater, Florida.

Kazeto Y, Kohara M, Miura T, Miura C, Yamaguchi S, Trant JM, Adachi S, Yamauchi K 2008 Japanese eel follicle-stimulating hormone (Fsh) and luteinizing hormone (Lh): production of biologically active recombinant Fsh and Lh by Drosophila S2 cells and their differential actions on the reproductive biology. *Biology of Reproduction* **79** 938-946.

Koulish S, Kramer CR, Grier HJ 2002 Organization of the male gonad in a protogynous fish, *Thalassoma bifasciatum* (Teleostei: Labridae). *Journal of Morphology* **254** 292-311.

Krietsch T, Fernandes MS, Kero J, Losel R, Heyens M, Lam EW-F, Huhtaniemi I, Brosens JJ, Gellersen B 2006 Human homologs of the putative G protein-coupled membrane progestin receptors (mPR*α*, *β*, and *γ*) localize to the endoplasmic reticulum and are not activated by progesterone. *Molecular Endocrinology* **20** 3146-3164.

Leal MC, Cardoso ER, Nóbrega RH, Batlouni SR, Bogerd J, França LR, Schulz RW 2009 Histological and stereological evaluation of zebrafish (*Danio rerio*) spermatogenesis with an emphasis on spermatogonial generations. *Biology of Reproduction* **81** 177-187.

Leung PC, Steele GL 1992 Intracellular signaling in the gonads. *Endocrine reviews* **13** 46-98.

Lösel R, Wehling M 2003 Nongenomic actions of steroid hormones. *Nature Reviews Molecular Cell Biology* **4** 46-55.

Matta SL, Vilela DA, Godinho HP, França, LR 2002 The goitrogen 6-n-propyl-2-thiouracil (PTU) given during testis development increases Sertoli and germ cell numbers per cyst in fish: the tilapia (*Oreochromis niloticus*) model. *Endocrinology* **143** 970-978.

Miura C, Ohta T, Ozaki Y, Tanaka H, Mirua T 2009 Trypsin is a multifunctional factor in spermatogenesis. *Proceedings of the National Academy of Sciences of the United States of America* **106** 20972-20977.

Miura T, Higuchi M, Ozaki Y, Ohta T, Miura C 2006 Progestin is an essential factor for the initiation of the meiosis in spermatogenetic cells of the eel. *Proceedings of the National Academy of Sciences of the United States of America* **103** 7333-7338.

Miura T, Miura C, Ohta T, Nader MR, Todo T, Yamauchi K 1999 Estradiol-17*β* stimulates the renewal of spermatogonial stem cells in males. *Biochemical and Biophysical Research Communications* **144** 5504-5510.

Miura T, Yamauchi K, Takahashi H, Nagahama Y 1991a Hormonal induction of all stages of spermatogenesis in vitro in the male Japanese eel (*Anguilla japonica*). *Proceedings of the National Academy of Sciences of the United States of America* **88** 5774-5778.

Miura T, Yamauchi K, Takahashi H, Nagahama Y 1991b Involvement of steroid hormones in gonadotropin-induced testicular maturation in male Japanese eel (*Anguilla japonica*). *Biomedical Research* **12** 241-248.

Miura T, Yamauchi K, Takahashi H, Nagahama Y 1992 The role of hormones in the acquisition of sperm motility in salmonid fish. *The Journal of Experimental Zoology* **261** 359-363.

Mukherjee A, Park-Sarge OK, Mayo KE 1996 Gonadotropins induce rapid phosphorylation of the 3',5'-cyclic adenosine monophosphate response element bingding protein in ovarian granulose cells. *Endocrinology* **137** 3234-3235.

Oba Y, Hirai T, Yoshiura Y, Yoshikuni M, Kawauchi H, Nagahama Y 1999a Cloning, functional characterization, and expression of a gonadotropin receptor cDNA in the ovary and testis of amago salmon (*Oncorhynchus rhodurus*). *Biochemical and Biophysical Research Communications* 263 584-590.

Oba Y, Hirai T, Yoshiura Y, Yoshikuni M, Kawauchi H, Nagahama Y 1999b The duality of fish gonadotropin receptors: cloning and functional characterization of a second gonadotropin receptor cDNA expressed in the ovary and testis of amago

salmon (*Oncorhynchus rhodurus*). *Biochemical and Biophysical Research Communications* **265** 366-371.

Ohta T, Miyake H, Miura C, Kamei H, Aida K, Miura T 2007 Follicle-stimulating hormone induces spermatogenesis mediated by androgen production in Japanese eel, *Anguilla japonica. Biology of Reproduction* **77** 970-977.

Petersen C, Soder O 2006 The Sertoli cell—a hormonal target and 'super' nurse for germ cells that determines testicular size. *Hormone Research* **66** 153-161.

Plant TM, Marshall GR 2001 The functional significance fo FSH in spermatogenesis and the control of its secretion in male primates. *Endocrine Reviews* **22** 764-786.

Pudney J 1993 Comparative cytology of the non-mammalian vertebrate Sertoli cell. In: Russell LD and Griswold MD (Eds.), *The Sertoli Cell*. pp. 611–657. Cache River Press, Clearwater, Florida.

Pudney J 1995 Spermatogenesis in nonmammalian vertebrates. *Microscopy Research and Technique* **32** 459-497.

Sambroni E, LeGac F, Breton B, Lareyre JJ 2007 Functional specificity of the rainbow trout (*Oncorhynchus mykiss*) gonadotropin receptors as assayed in a mammalian cell line. *Journal of Endocrinology* **195** 213-228.

Sampath Kumar R, Ijiri S, Trant JM 2000 Molecular biology of channel catfish gonadotropin receptors: 1. Cloning of a functional luteinizing hormone receptor and preovulatory induction of gene expression. *Biology of Reproduction* **64** 1010-1018.

Schulz RW, Menting S, Bogerd J, Franca LR, Vilela DAR, Godinho HP 2005 Sertoli cell proliferation in the adult testis: evidence from two fish species belonging to different orders. *Biology of Reproduction* **73** 891-898.

Schulz RW, de França LR, Lareyre JJ, LeGac F, Chiarini-Garciac H, Nobrega RH, Miura T 2010 Spermatogenesis in fish. *General and Comparative Endocrinology* **165** 390-411.

Scott AP, Sumpter JP 1989 Seasonal variations in testicular germ cell stages and in plasma concentrations of sex steroids in male rainbow trout (*Salmo gairdneri*) maturing at 2 years old. *General and Comparative Endocrinology* **73** 46-58.

So WK, Kwok HF, Ge W 2005 Zebrafish gonadotropins and their receptors: II. Cloning and characterization of zebrafish follicle-stimulating hormone and luteinizing hormone subunits: their spatial-temporal expression patterns and receptor specificity. *Biology of Reproduction* **72** 1382-1396.

Song M, Gutzeit HO 2003 Effect of 17α-ethynylestradiol on germ cell proliferation in organ and primary culture of medaka (*Oryzias latipes*) testis. *Development, growth & differentiation* **45** 327-337.

Thomas P 2008 Characteristics of membrane progestin receptor alpha (mPRα) and progesterone membrane receptor component 1 (PGMRC1) and their roles in mediating rapid progestin actions. *Frontiers in Neuroendocrinology* **29** 292-312.

Ueda H, Kanbegawa A, Nagahama Y 1985 Involvement of gonadotrophin and steroid hormones in spermiation in the amago salmon, *Oncorhynchus rhodurus*, and

goldfish, *Carassius auratus*. *General and Comparative Endocrinology* **59** 24-30.

Vizziano D, LeGac F, Fostier A 1996a Effect of 17*β*-estradiol, testosterone, and 11-ketotestosterone on 17*α*,20*β*-dihydroxy-4-pregnen-3-one production in the rainbow trout testis. *General and Comparative Endocrinology* **104** 179-188.

Vizziano D, Fostier A, Le Gac F, Loir M 1996b 20*β*-hydroxysteroid dehydrogenase activity in nonflagellated germ cells of rainbow trout testis. *Biology of Reproduction* **54** 1-7.

Chapter 2

二

Molecular Cloning and Functional Characterization of a Zebrafish Nuclear Progesterone Receptor

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ABSTRACT

Progestagenic sex steroid hormones play critical roles in reproduction across vertebrates, including teleost fish. To further our understanding of how progesterone modulates testis functions in fish, we set out to clone a progesterone receptor (*pgr*) cDNA exhibiting nuclear hormone receptor features from zebrafish testis. The open-reading frame of *pgr* consists of 1854 bp, coding for a 617 amino acids long protein showing highest similarity with other piscine Pgr proteins. Functional characterization of the receptor expressed in mammalian cells revealed that zebrafish Pgr exhibited progesterone-specific, dose-dependent induction of reporter gene expression, with 17*α*,20*β*-dihydroxy- 4-pregnen-3-one (DHP), a typical piscine progesterone, showing the highest potency. Expression of *pgr* mRNA: (i) appeared in embryos at 8 hours post fertilization; (ii) was significantly higher in developing ovary than in early transforming testis at 4 weeks of age but vice versa in young adults at 12 weeks of age and thus resembling the expression pattern of the germ cell marker *piwil1*; and, (iii) was restricted to Leydig and Sertoli cells in adult testis. Zebrafish testicular explants released DHP concentration-dependently in response to high concentrations of recombinant zebrafish gonadotropins. In addition, DHP stimulated 11-ketotestosterone release from zebrafish testicular explants, but only in the presence of its immediate precursor 11*β*-hydroxytestosterone. This stimulatory activity was blocked by a Pgr antagonist (RU486), suggesting that 11*β*-hydroxysteroid dehydrogenase activity was stimulated by DHP via Pgr. Our data suggest that DHP contributes to the regulation of Leydig cell steroidogenesis and potentially – via Sertoli cells – also of germ cell differentiation in zebrafish testis.

INTRODUCTION

Progestagenic sex steroid hormones play critical roles in vertebrate reproduction. In mammals, progesterone signaling regulates multiple reproductive processes in females, including follicle growth, oocyte maturation, ovulation, implantation, and the maintenance of pregnancy [1]. In male mice, however, loss of progesterone receptor (PGR) function does not result in a testis phenotype and the animals are fertile although plasma LH levels are higher than normal [2], reflecting a negative feedback effect of progesterone on LH release that is used in hormonal male contraception [3]. It has also been reported that progesterone stimulates the acrosome reaction [4, 5].

In many teleost fish, the biologically active progesterone molecule is 17*α*,20*β*-dihydroxy-4-pregnen-3-one (DHP), which plays crucial roles during the resumption of meiosis in final oocyte maturation [6]. However, also in male fish DHP plays multiple and significant roles in reproductive physiology. Plasma DHP levels increase during the reproductive cycle [7, 8] when germ cells enter into meiosis, and - in a later stage - when attaining full maturity and spawning activity. Studies on testicular steroid metabolism in rainbow trout showed that the DHP precursor 17*α*-hydroxy-4-pregnen-3-one (17*α*(OH)P4) is efficiently converted to DHP during three periods, namely when testis tissue was immature and contained spermatogonia only, when germ cells entered meiosis, and in fully mature fish [9]. As regards the final stages of sperm maturation, DHP has been reported to stimulate sperm hydration

[10] and acquisition of sperm motility [11, 12]. More recently, it was found that DHP induces the entry of male germ cells into meiosis [13]. Finally, DHP is a highly potent pheromone in fish [14, 15]. Therefore fish are an interesting vertebrate group to study the spectrum of progesterone actions in male reproduction.

The biological activity of progesterone is mediated via specific receptors. A single hormone can interact with different receptor types. For estrogens [16], retinoids [17], or prostaglandins [18], it is known that, next to members of the nuclear receptor family, there are also membrane-associated receptors. Also for progesterones, different receptor types have been reported, either belonging to the nuclear hormone receptor superfamily, or to the membrane-associated receptor family [19, 20], although the functions mediated by membrane-associated progesterone receptors are still a matter of discussion [21]. The present study deals with the nuclear progesterone receptor of zebrafish.

Zebrafish (*Danio rerio,* Cyprinidae) is a vertebrate model system offering the attractive combination of being simple to maintain and suitable for studies on development, genetics, diseases, and physiology [22, 23]. Zebrafish are also used for basic [23, 24] and applied [25, 26, 27] studies on the biology of reproduction. Recently, we presented a detailed and quantitative description of testis structure and the different stages of germ cell development during spermatogenesis [28]. To develop our understanding of the two main testicular functions, spermatogenesis and steroidogenesis, and to elucidate the possible Pgr role(s) in this context, we set out to clone the zebrafish nuclear progesterone receptor (*pgr*) cDNA. We report the pharmacological characterization of the zebrafish Pgr, *pgr* mRNA expression patterns during ontogenesis, and in different adult tissues. We also studied the capacity of zebrafish testicular explants to produce DHP under gonadotropin stimulation and the ability of DHP to modulate androgen release in a Pgr-dependent manner.

MATERIAL AND METHODS

Animals and source of steroid hormones

Tübingen AB strain zebrafish, outbred zebrafish from a mixed background, or transgenic zebrafish (AB background) expressing enhanced green fluorescent protein under the control of the germ cell-specific *vas* promoter (*vas::egfp*) [29], were used. Animal culture [30] and experimentation were consistent with Dutch regulations and were approved by the Utrecht University Life Sciences Committee for Animal Care and Use. Under the conditions of constant photoperiod and temperature in our aquarium facility, we see no evidence for a seasonality of reproductive parameters [28].

The following steroids were used in the current study: DHP, 17α,20β,21-trihydroxy-4-pregnen-3-one (20β-S), progesterone (P4), 17α(OH)P4, testosterone (T), 11-ketotestosterone (11-KT), 17β-estradiol (E_2), cortisol, 11β-hydroxytestosterone (11β-OHT), synthetic progestin promegestone (R5020), and mifepristone (RU486). All steroids were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands) except for R5020 that was obtained from Perkin Elmer (Waltham, MA, USA).

Cloning and sequence analysis of zebrafish *pgr* cDNA

Total RNA was extracted from adult zebrafish testes using the FastRNA Pro Green kit (MP Biomedicals, Solon, OH, USA). Poly(A)-rich zebrafish testis RNA was isolated using Dynabeads-oligo dT_{25} (Dynal A.S., Oslo, Norway) and reverse transcribed to 5'- and 3'-RACE ready cDNA using a SMART RACE cDNA amplification kit (Clontech, Mountain View, CA, USA) following the manufacturers' instructions.

Two partial zebrafish *pgr* cDNAs (GenBank accession numbers: DQ017620 and XM_001343705) were obtained from GenBank by BLAST searches [31], using the human *PGR* cDNA sequence (GenBank accession number: M15716) as query sequence. Based on these two sequences, a full-length zebrafish *pgr* cDNA sequence was predicted, which was confirmed with BLAST searches in the *Danio rerio* Ensembl database (http://www.ensembl.org). To generate a zebrafish *pgr* expression vector construct, the predicted *pgr* open-reading frame (ORF) was PCR amplified using primers overlapping the start and stop codons (2783, 5'-TTGCCACCATGGACACGGTGAACACTTCTCCCGCTGATT-3'; 2784, 5'-TCGTCCGGTCGGCCTTCATTTGTGGTGA-3'), cloned into pcDNA3.1/V5-His TOPO (Invitrogen, Carlsbad, CA, USA) in the correct orientation, and sequence verified using Dye Terminator cycle sequencing chemistry (Applied Biosystems; Foster City, CA, USA).

After obtaining the zebrafish *pgr* cDNA sequence, a homology search was performed using the BLAST program [31]. 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 W (PAM250) algorithm [32], and percentages identity were calculated. The percentage identity is a measure of similarity between the zebrafish and other PGR sequences, derived by taking the matches over the matches, mismatches and gaps, according to the formula: similarity = (100 × consensus length) / (consensus length + mismatches + gaps). For comparison with the zebrafish Pgr, we only selected (deduced) PGR amino acid sequences from studies that experimentally demonstrated progesterone binding to the receptors; the respective GenBank accession numbers are available as supplemental data. A phylogenetic tree was constructed from the aligned sequences using the neighbor-joining method [33].

Transactivation assays for zebrafish Pgr

HEK 293T cells were used to express the zebrafish Pgr protein. Cells were seeded in 10 cm dishes (~2×10^6 cells per dish) in Dulbecco modified Eagle medium (DMEM) supplemented with 10% v/v fetal bovine serum (FBS), glutamine, and penicillin/streptomycin (Gibco, Breda, The Netherlands) at 37°C in a 5% CO_2 incubator. After 24 h, the cells were co-transfected using a standard calcium phosphate precipitation method [34] with 400 ng of the zebrafish *pgr* expression plasmid and 7 μg of pGL3-MMTV-Luc plasmid, containing the mouse mammary tumor virus-long terminal repeat (MMTV-LTR) and the *Photinus pyralis* luciferase

gene [35]. After 5-6 h, the transfected cells were transferred to 24-well plates coated with poly-L-lysine hydrobromide (Sigma-Aldrich). 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 duplicates) with final concentrations ranging between 0.1 nM and 10 μM (n=2 per condition tested; see Fig. 2) or with different concentrations of DHP (10 pM to 1 μM) in the presence of the mammalian PGR antagonist RU486 (1 to 100 μM; see Fig. 8) [36]. After incubation at 37°C for 24–36 h, the cells were harvested in lysis mix (100 mM potassium phosphate buffer pH 7.7, 1% v/v Triton X-100 [Sigma-Aldrich], 15% v/v glycerol, and 2 mM dithiotreitol [DTT]) and stored at –80°C. Luciferase activity was determined by adding an equal volume of substrate mix (100 mM potassium phosphate buffer pH 7.7, 250 mM D-luciferin [Invitrogen], 1 mM DTT, 2 mM ATP [Roche, Woerden, The Netherlands] and 15 mM magnesium sulfate [Promega, Leiden, The Netherlands]) to thawed samples and luminescence was measured in a Packard TopCount NXT luminometer (Perkin Elmer Life Sciences, Meriden, CT, U.S.A.). Each compound was tested in three independent experiments using cells from different transfections.

Tissue and ontogenic analysis of zebrafish *pgr* mRNA expression

First, relative zebrafish *pgr* mRNA expression levels were examined in different organs obtained from adult zebrafish (n=3 individuals per sex). Second, changes in zebrafish *pgr* mRNA expression were analyzed from 0 to 24 hours post fertilization (hpf) in whole zebrafish embryos (n=3 pools of 20 embryos for each time point) to investigate whether *pgr* mRNA is among the maternally contributed mRNAs or when *pgr* mRNA expression starts during early embryonic development. Finally, relative gonadal *pgr* mRNA expression levels were studied during zebrafish sex differentiation. Zebrafish is an "undifferentiated" gonochoristics species, *i.e.* gonads initially develop as ovaries at ~2-3 weeks post fertilization (wpf) but, in future males, ovarian tissue soon degenerates and gonadal tissue transforms into testis starting at ~3-5 wpf [25, 37]. Using gonad samples from *vas::egfp* zebrafish to sort for testicular and ovarian tissue [29], we selected three sampling points during the ovary-to-testis transformation process: (1) initial phases of the sex reversal process at 4 wpf (fish at this age were classified as developing females or transforming males) [26, 37], (2) completion of testicular differentiation and start of meiosis/spermiogenesis at 8 wpf, and (3) young adults at 12 wpf. In order to investigate the relative *pgr* mRNA expression levels in relation to early stages of germ cell development, we also examined the relative expression levels of the germ cell marker *piwil1* [38] and of the meiosis-specific marker *sycp3l* [39].

Depending on the size of the tissue samples, either the FastRNA Pro Green kit (MP Biomedicals, Solon, OH, USA) or the RNAqueous®-Micro Kit (Ambion, Austin, TX, USA) was used for total RNA extraction. Synthesis of cDNA from total RNA samples was performed as described previously [40]. Primers to detect zebrafish *pgr*, *piwil1* and *sycp3l* mRNA were designed and tested before use for specificity and amplification efficiency on serial dilutions of testis cDNA (Supplemental Table 1) as

described elsewhere [40]. Primers and a 6-carboxy-fluorescein labelled probe were acquired to detect the endogenous control, *18S rRNA* (TaqMan® gene expression assays; Applied Biosystems). All real-time, quantitative PCR (qPCR) were performed in 20 µl reactions and C_t values determined in a 7900HT Real-Time PCR System (Applied Biosystems) using default settings, as described previously [35]. Relative *pgr*, *piwil1* and *sycp3l* mRNA levels were calculated as reported [40].

Cellular localization of *pgr* expression in zebrafish testis

The localization of *pgr* mRNA expression in zebrafish testis was investigated by in situ hybridization, and by qPCR analysis of laser micro-dissected testis tissue fractions and of testis tissue samples from germ cell-depleted, homozygous *piwil1* mutants [38].

A zebrafish *pgr*-specific PCR product was generated with primers 2737 (5'-GGGCGGGTGTTATTAACCCTCACTAAAGGGCTTGAAGAGTCAAACACA GTTTGATG-3') and 2738 (5'-CCGGGGGGTGTAATACGACTCACTATAGGGACTGATTCTAATTCTTTCTC CACTCTCTGAA-3'), which contained the T3 or T7 RNA polymerase promoter sequence (underlined) attached at their 5'-ends, respectively. The ~465 bp PCR product obtained was gel purified and served as template for digoxigenin-labeled cRNA probe synthesis, as described previously [41]. In situ hybridization was performed on 10 µm thick cryosections from adult zebrafish testis as reported previously [35], except that a 48 hours hybridization period was used.

Laser microdissection of zebrafish testis sections was carried out similar to the procedure described recently for African catfish (*Clarias gariepinus*) testis [42]. In brief, two testis tissue fractions were microdissected from freshly obtained cryosections and collected using a PALM® MicroBeam Instrument (PALM Microlaser Technologies, Bernried, Germany): interstitial tissue, identified by means of the 3*β*-hydroxysteroid dehydrogenase (3*β*-HSD) staining of Leydig cells, and intratubular tissue, containing spermatogenic cysts (germ/Sertoli cells units) with germ cells at all three major stages of spermatogenesis (mitotic, meiotic, and spermiogenic phase). Total RNA extraction of laser-microdissected samples (RNAqueous-Micro Kit, Ambion), linear amplification (MessageAmpTM II aRNA Amplification Kit, Ambion), and reverse-transcription to cDNA were performed as reported previously [42]. The relative *pgr* mRNA expression levels were quantified in zebrafish interstitial and intratubular tissue fractions. In this RNA amplification technique, poly(A)$^+$ mRNA is reverse-transcribed and converted into double stranded cDNA using an oligo(dT) primer containing a promoter for T7 RNA polymerase. The second strand cDNA serves as a transcription template for amplified antisense RNA (aRNA) production. Therefore, the target amplicons for *pgr* and *actb1* were designed in their last exons. Primers to detect the endogenous control *actb1* mRNA as well as the *pgr* mRNA were tested before use for specificity and amplification efficiency on serial dilutions of testis cDNA as described above (Supplemental Table 1) [40], while the RNA samples were DNAse I-treated before cDNA synthesis.

Homozygous *piwil1* mutant zebrafish [*piwil1*$^{(-/-)}$] have germ cell-depleted testes

[38]. The relative zebrafish *pgr* mRNA expression levels were compared between testis of *piwil1*$^{(-/-)}$ and wild-type zebrafish by qPCR. Total RNA extraction and reverse transcription to cDNA were performed as described above, and *18S rRNA* served as an endogenous control gene in this series.

Short-term in vitro steroid secretion by zebrafish testicular explants

Testicular tissue explants from sexually mature, outbred zebrafish were used in the experiments described below. Both testes from six fish were used per condition to be tested. For each individual, one testis served as control for the contralateral one, as described previously [43], hence representing biologically independent sample sets. Moreover, two series of similar experiments were carried out. Incubations lasted 18 h in a humidified air atmosphere at 25ºC in 96-well flat-bottom plates (Corning Inc., New York, USA) using a final volume of 200 µl. Basal culture medium consisted of 15 g/L Leibovitz L-15 (Invitrogen) supplemented with 10 mM Hepes (Merck), 0.5% w/v bovine serum albumin fraction V (Roche, Mannheim, Germany), 0.4 mg/L amphotericine B (Fungizone®; Invitrogen) and 200,000 U/L penicillin/streptomycin (Invitrogen); pH was adjusted to 7.4. The different solvents used (DMSO <0.5 %; PBS < 0.4%; ethanol < 0.001%) for different test substances always were identical between control and treated testes, and the different solvents had no significant effect on basal steroid release (see below). After incubation the tissue explants were weighed. The medium was heated at 80°C for 1 h, centrifuged for 30 min (16,000 g) and the supernatant collected and stored at -20°C until quantification of levels of different steroids by radioimmunoassay [44]. The results are expressed as amount of steroid released into the medium per milligram of testis tissue incubated.

First, testicular explants were challenged with increasing concentrations of single chain recombinant zebrafish Fsh (rec-zfFsh; from 50 to 1000 ng/ml), single chain recombinant zebrafish Lh (rec-zfLh; from 100 to 2000 ng/ml) or the adenylate cyclase activator forskolin (from 0.1 to 25 µM; Sigma-Aldrich). Details on the production and purification by affinity-chromatography of recombinant zebrafish gonadotropins will be published separately. Gonadotropin stocks were prepared in PBS, while the forskolin stock was prepared in DMSO. After incubation, DHP levels in the medium were quantified by radioimmunoassay [44]. Significant differences among the different concentrations of each test substance were identified by one way ANOVA followed by the Student-Newman-Keuls test. DHP release in basal medium and in media containing low gonadotropin concentrations were below the detection limit of the assay (4 pg/50 µl) and were excluded from the statistical analyses.

Second, the ability of DHP to stimulate 11-KT production by zebrafish testis tissue was studied by incubating testicular explants with either DHP (100 ng/ml), 11β-OHT (10 ng/ml), or DHP plus 11β-OHT. Steroid stocks were prepared in ethanol. Our previous studies have shown that the main steroidogenic pathway in zebrafish testis leads from the conversion of 11β-hydroxyandrostenedione to 11-ketoandrostenedione, catalyzed by 11β-hydroxysteroid dehydrogenase (11β-HSD), followed by conversion of 11-ketoandrostenedione to 11-KT, mediated by 17β-hydroxysteroid dehydrogenase [35]. To circumvent this main steroidogenic pathway, we used 11β-OHT as substrate,

which can be converted to 11-KT by 11β-HSD. After incubation, 11-KT levels in the medium were quantified by radioimmunoassay [44]. Because of the experimental design (incubation of one testis under basal conditions, the contralateral one under experimental conditions), we obtained a basal 11-KT release data set for each condition assayed. Homogeneity of basal androgen release among the different groups of replicates was tested by one way ANOVA. No significant differences were identified (P>0.05) and therefore, basal release data were compiled into one single basal 11-KT release condition. Thereafter, significant differences among the different treatments were identified by one way ANOVA followed by the Student-Newman-Keuls test (P<0.05).

Effects of RU486 on DHP-stimulated 11-KT release

To investigate if the DHP-stimulated 11-KT production was Pgr-dependent, we incubated testicular explants with DHP (100 ng/ml) and 11β-OHT (10 ng/ml) or with DHP and 11β-OHT together with RU486 (10 μM). This concentration of RU486 was chosen because it partially inhibited DHP-stimulated and Pgr-mediated reporter gene expression, while it did not inhibit androgen production in the presence of a concentration of rec-zfLh (500 ng/ml) that stimulated 11-KT release but not yet DHP release.

RESULTS

Isolation and sequence analysis of zebrafish *pgr* cDNA

The open-reading frame (ORF) of the zebrafish *pgr* consisted of 1854 nucleotides (GenBank accession number: FJ409244), encoding a protein of 617 amino acids (Supplemental Fig. 1). The comparison of the deduced amino acid sequence of the zebrafish Pgr with PGRs from other species is shown in Supplemental Table 2. The zebrafish Pgr sequence could be subdivided into 4 domains. An N-terminal transactivation domain showed low homology (7.1-23.9%), while the putative DNA-binding domain (DBD) and ligand-binding domain (LBD) showed high homology (DBD, 83.3–97.2%; LBD, 65.3–83.2%), with PGRs of other vertebrates. The overall homology of zebrafish Pgr with PGRs from other species is 43.6-66.3%.

A phylogenetic tree, constructed from the aligned amino acid sequences using the neighbor-joining method [33], revealed that the known PGRs are divided into three major clades (Fig. 1). The first clade consisted of fish Pgrs, the second clade contained avian, reptilian, and amphibian PGRs, and the last clade contained mammalian PGRs.

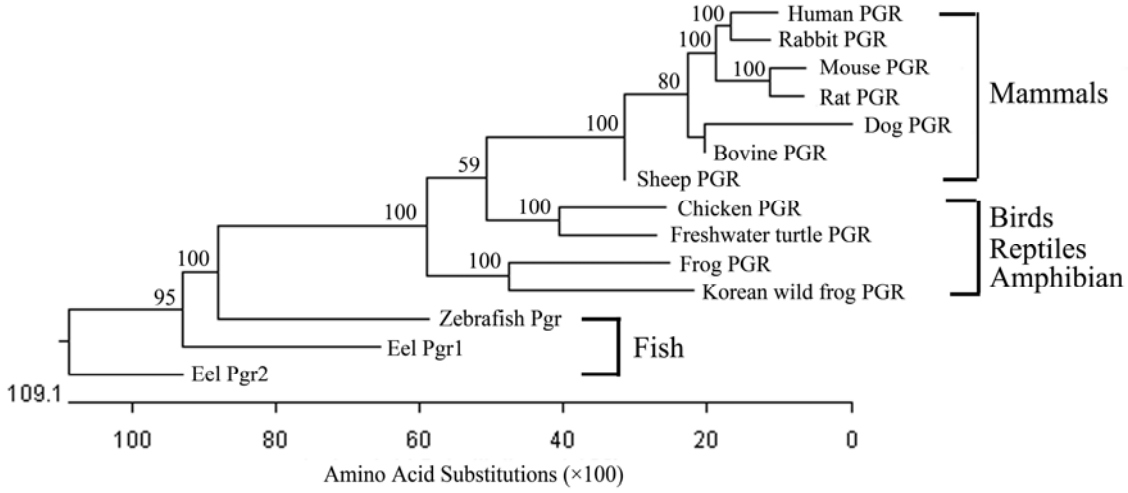

Figure 1 Phylogenetic tree of PGRs. The Jotun Hein method was used to perform multiple sequence alignment. The phylogenetic tree was constructed by the neighbor-joining method using the MegAlign program (Lasergene software package; DNASTAR Inc.), including only sequences where progesterone binding had been demonstrated experimentally. The horizontal distances to the branching points are proportional to the number of amino acid substitutions. The numbers beside the branches indicate bootstrap values from 1000 replicates.

Steroid-specific transactivation of the zebrafish Pgr

To determine the steroid-dependent transactivation properties of the zebrafish Pgr, HEK 293T cells were transfected with the pGL3-MMTV-Luc reporter construct alone or together with the zebrafish *pgr* expression vector construct. Next, transfected cells were stimulated with increasing concentrations of different steroid hormones. When transfected with the empty vector, none of the steroid hormones tested [i.e. DHP, 20β-S, P4, 17α(OH)P4 and R5020] increased luciferase activity (data not shown), indicating that HEK 293T cells do not express an endogenous PGR. Dose-dependent,

Pgr-mediated activation of the MMTV promoter was shown for all aforementioned progesterone-related steroids (Fig. 2A), the one with the lowest EC_{50} value being DHP (8.0±1.3 nM). Also at a fixed concentration of 1 μM, DHP was the most potent inducer of luciferase activity (69-fold above control; Fig. 2B). The other four progesterone-related hormones tested elicited significant increases of luciferase activity as well (from 26- to 53-folds above control), while other steroid hormones assayed (T, 11-KT, E_2, or cortisol) were ineffective at the dose tested (1 μM).

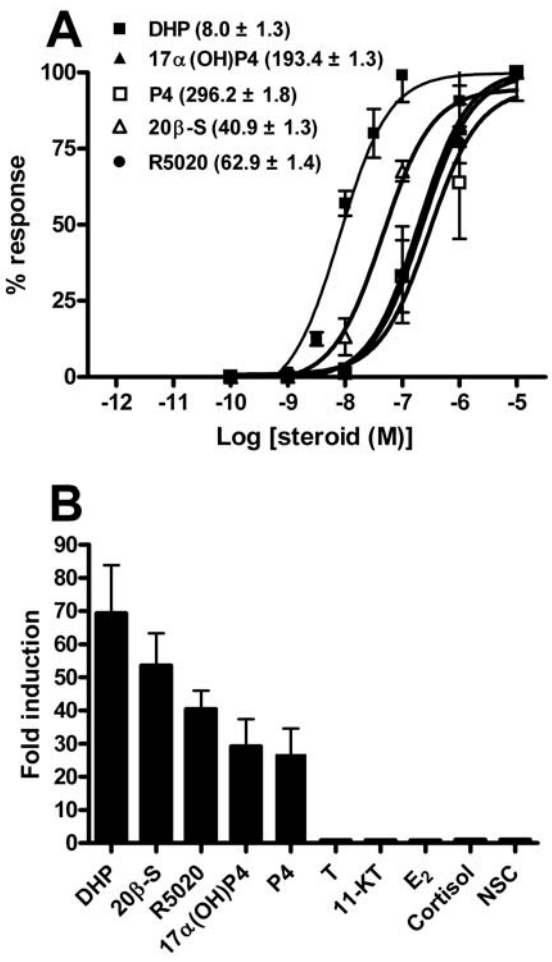

Figure 2 Ligand-induced transactivation properties of the zebrafish Pgr. HEK 293T cells were transiently co-transfected with the pGL3-MMTV-Luc vector and the zebrafish *pgr* expression vector construct. (A) Transfected cells were incubated with increasing concentrations of various progesterones (from 0.1 nM to 10 μM). Percentage (%) of response: values are given relative to the maximal amount of luciferase activity for each condition. Each point represents the mean ± SEM of three independent experiments, with duplicates for each steroid concentration. The EC_{50} (nM) value of each progesterone is given between brackets. Curves were generated using non-linear regression (GraphPad Prism 4.0). (B) Transfected cells were incubated with or without 1 μM of the steroids indicated. Data are expressed as the ratio of steroid:NSC (no steroid control). Each column represents the mean ratio of three independent experiments, with the vertical bar representing the SEM, if not too small for the scale.

Tissue distribution of zebrafish *pgr* mRNA

Real-time, quantitative PCR analysis of several tissues from adult male and female zebrafish showed that *pgr* mRNA was predominantly expressed in the testis (Fig. 3). Significantly lower *pgr* mRNA levels were found in the ovary and in most other tissues tested, without showing significant differences between sexes. Heart and gill tissue did not express detectable *pgr* mRNA level.

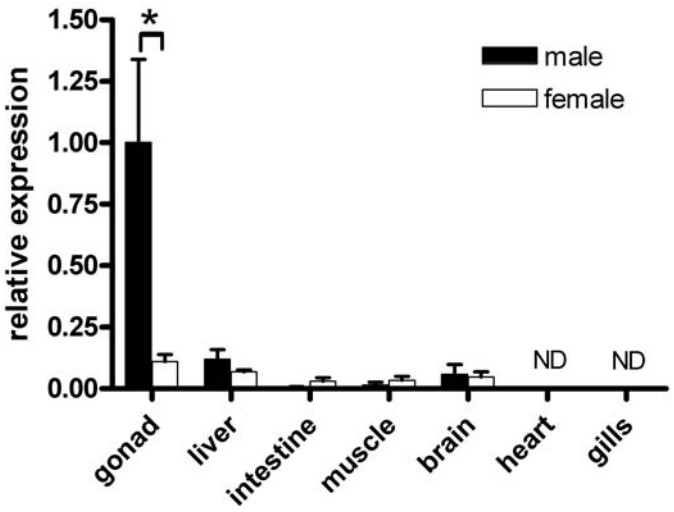

Figure 3 Relative expression of zebrafish *pgr* mRNA in adult organs. Total RNA was extracted from various tissues of male (black columns) and female (white columns) zebrafish. The expression level was normalized to the expression of *18S rRNA*. Values represent mean ± SEM (n=3) relative to testicular *pgr* mRNA levels. The asterisk indicates a significant difference between testicular and ovarian (P<0.05) tissue (Student's t-test). ND, not detectable.

Ontogenic analysis of zebrafish *pgr* mRNA relative expression

Ontogenic changes in zebrafish *pgr* mRNA expression were analyzed during early embryogenesis in whole embryos, and in sex-differentiating and sexually mature gonads by qPCR. Analysis of zebrafish embryos showed that *pgr* mRNA became detectable from 8 hpf onwards (Fig. 4A), i.e. there was no maternal contribution of *pgr* mRNA. Expression analysis in early sex-differentiating gonads at 4 wpf revealed that *pgr* mRNA expression was significantly higher in ovarian than in testicular tissue. At 8 wpf, when sex differentiation is completed and pubertal gonad development has started, *pgr* mRNA is increased more than 20-fold and showed similar levels in both sexes (Fig. 4B). High testicular expression levels were maintained in young adults (12 wpf), while ovarian *pgr* mRNA levels decreased significantly compared to ovaries at 8 wpf.

In addition, the expression levels of the specific germ-cell transcripts *piwil1* (predominantly expressed during the mitotic and early meiotic germ cell stages [38]) and *sycp3l* (exclusively expressed in meiotic cells [39]) were quantified during zebrafish sex differentiation. The expression pattern of *piwil1* mRNA was similar to that observed for *pgr* mRNA (Fig. 4C). Gonadal *sycp3l* mRNA expression showed similarly low levels in both sexes at 4 wpf (Fig. 4D). At 8 and 12 wpf *sycp3l* mRNA amounts increased significantly in both testes and ovaries although the levels measured in testis tissue were ~20-fold higher than in ovarian tissue (P<0.01).

Cellular localization of *pgr* mRNA expression in zebrafish testis

Identification of specific cell types expressing the zebrafish *pgr* mRNA was accomplished by in situ hybridization using zebrafish testis cryosections. A strong signal was observed in Leydig cells in addition to a weaker signal in Sertoli cells (Fig. 5A). No signal was observed when adjacent sections were hybridized with the sense cRNA *pgr* probe (Fig. 5A).

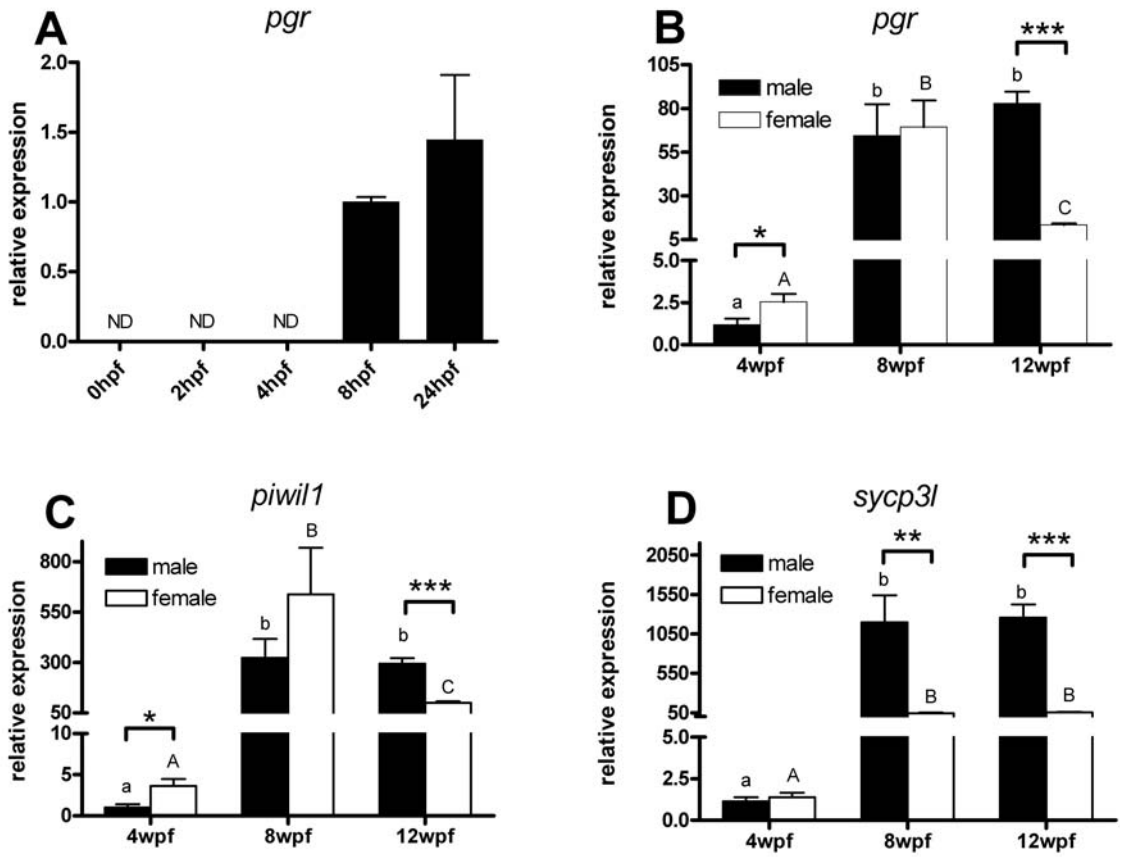

Figure 4 The relative expression of zebrafish *pgr* (A, B), *piwil1* (C) and *sycp3l* (D) mRNAs during ontogeny. (A) RNA was extracted from whole embryos at different stages of development. The level of expression was determined by qPCR and normalized to the expression of *18S* rRNA. Data are expressed as mean ± SEM (n=3) relative to *pgr* mRNA levels in 8hpf embryo. ND, not detectable. (B, C, D) RNA was extracted from developing gonads of individual *vas::egfp* transgenic zebrafish and classified into testis or ovary according to their EGFP expression pattern. Relative levels of *pgr* (B), *piwil1* (C), and *sycp3l* (D) mRNA were determined by qPCR after normalization to the levels of *18S* rRNA. Data are expressed as mean ± SEM (n=6), relative to *pgr* mRNA levels in 4wpf male testis. The asterisks indicate a significant difference in relative expression between male and female (**P*<0.05, ***P*<0.01, ****P*<0.001). Bars marked with different letters are significantly different between each other (*P*<0.01; lower case for males, upper case for females).

Confirmation of the *pgr* mRNA expression by Sertoli cells was obtained by qPCR analysis of laser microdissected samples (Fig. 5B). The levels of *pgr* mRNA in the intratubular fraction were similar to those of the interstitial fraction. Sertoli cell expression in the intratubular fraction, and somatic cell expression in general, was further supported by analyzing *pgr* mRNA expression in testis samples of sterile *piwil1*$^{(-/-)}$ mutants. The *pgr* mRNA levels in the germ cell-free *piwil1*$^{(-/-)}$ testis were similar to those in wild-type testis (Fig. 5C), demonstrating that *pgr* mRNA in the intratubular fraction is associated with Sertoli cells, the only other intratubular cell type next to germ cells.

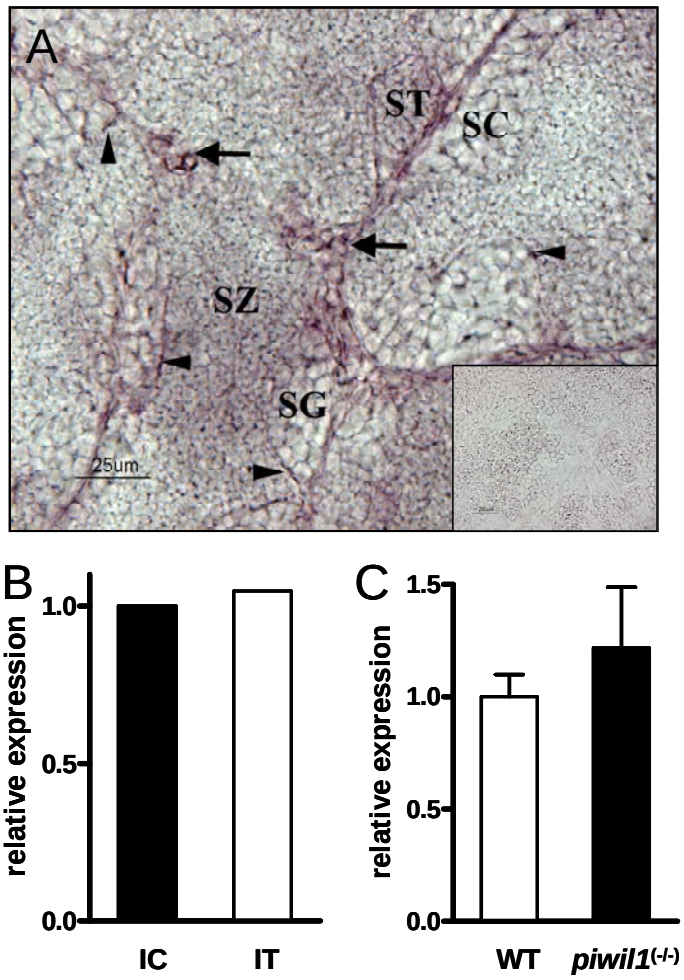

Figure 5 Cellular localization of *pgr* mRNA expression in zebrafish testis. (A) In situ hybridization for *pgr* mRNA on testis sections of sexually mature zebrafish. The antisense cRNA probe showed strong staining in Leydig cells (arrows) and weak staining in Sertoli cells (arrowheads). Germ cells (SG, spermatogonia; SC, spermatocytes; ST, spermatids; SZ, spermatozoa) were devoid of signal. Insert shows the sense cRNA probe; note the absence of specific staining. (B) Relative expression levels of zebrafish *pgr* from interstitial (IC) and intratubular (IT) tissue fractions. The level of *pgr* expression was normalized to the expression of *actb1*. (C) Relative expression levels of zebrafish *pgr* from wild-type (WT) and *piwil1* mutant [*piwil1*$^{(-/-)}$] testis. The level of *pgr* expression was normalized to the expression of *18S* rRNA.

Short-term in vitro steroid secretion by zebrafish testicular explants

The capacity of zebrafish testis tissue to release DHP when stimulated by zebrafish gonadotropins or the adenylate cyclase activator forskolin, was evaluated in overnight primary testis tissue cultures. DHP release under basal conditions as well as in the presence of low to intermediate concentrations of recombinant zebrafish gonadotropins was below the detection limit of the assay (4 pg/50 µl; Fig. 6). The lowest rec-zfFsh concentration eliciting a detectable DHP release was 100 ng/ml, whereas for rec-zfLh this concentration was 1000 ng/ml (Fig. 6A, B). Also at higher concentrations, rec-zfFsh was significantly more potent in stimulating DHP release than rec-zfLh (P<0.05). The profile of DHP release in the presence of increasing amounts of forskolin (Fig. 6C) showed a clear dose-dependency, and 0.1 µM forskolin induced the first significant DHP release response. The DHP release induced by 10 and 25 µM forskolin was not significantly different from that induced by 1000 ng/ml rec-zfFsh (P>0.05) and thus can be considered as the maximum response.

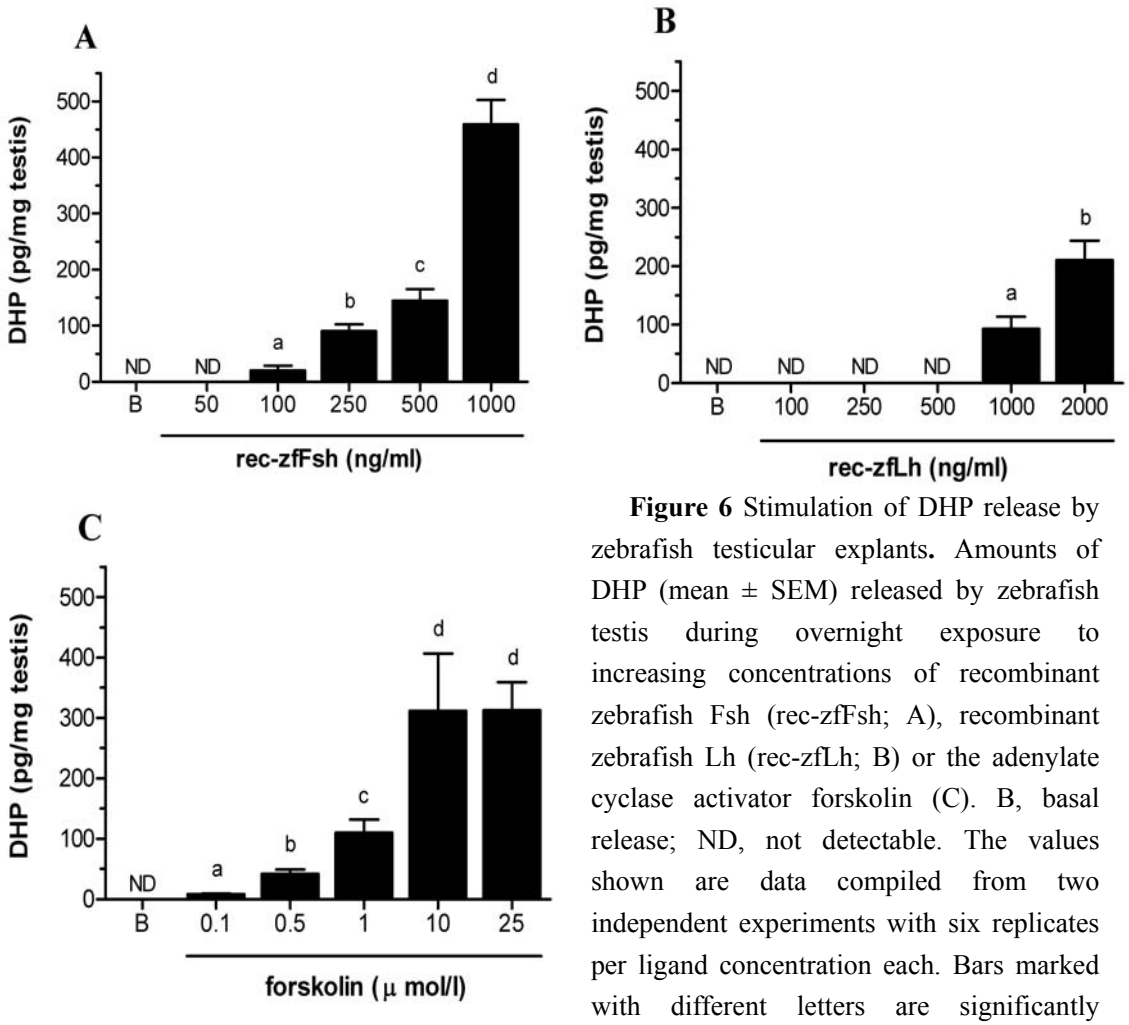

Figure 6 Stimulation of DHP release by zebrafish testicular explants**.** Amounts of DHP (mean ± SEM) released by zebrafish testis during overnight exposure to increasing concentrations of recombinant zebrafish Fsh (rec-zfFsh; A), recombinant zebrafish Lh (rec-zfLh; B) or the adenylate cyclase activator forskolin (C). B, basal release; ND, not detectable. The values shown are data compiled from two independent experiments with six replicates per ligand concentration each. Bars marked with different letters are significantly different from each other (P<0.05).

The ability of DHP to stimulate 11-KT release by zebrafish testicular explants was evaluated to test if an observation made in juvenile eel testis [45] also applied to adult zebrafish testis. Neither the presence of 100 ng/ml DHP nor 10 ng/ml 11β-OHT (a steroid precursor of 11-KT) alone increased the amount of 11-KT released compared with control (Fig. 7). However, when the testicular explants were incubated with both DHP and 11β-OHT, 11-KT production increased by 2.5-fold compared with control (P<0.001), suggesting that DHP is able to increases 11β-HSD activity.

Effects of RU486 on DHP-stimulated 11-KT release

Transactivation of the MMTV promoter via the DHP-stimulated zebrafish Pgr was inhibited by RU486. The antagonistic effect of RU486 on Pgr-mediated transactivation by increasing doses of DHP (10 pM to 1 μM) was reflected in 2- or 10-fold higher concentrations of DHP needed to reach half maximal reporter gene activation with DHP in the presence of 10 (EC_{50}=14 nM) or 100 μM (EC_{50}=60 nM) RU486 respectively, compared with the condition where no RU486 was included (Fig. 8A). Also, the luciferase activity induced by DHP (at 1 μM) was inhibited by RU486 (Fig. 8B).

Althought RU486 showed best inhibitory effect at 100 μM, we found at this concentration, it interfered with androgen production while 10 μM RU486 did not (data not shown). Therefore, We used RU486 at 10 μM to test if the ability of DHP to stimulate 11-KT production was Pgr-dependent. In the presence of RU486 (10 μM), the testicular 11-KT production induced by DHP plus 11β-OHT was significantly decreased (P<0.01) (Fig. 8C), suggesting that DHP increased 11β-HSD activity via a Pgr-dependent mechanism.

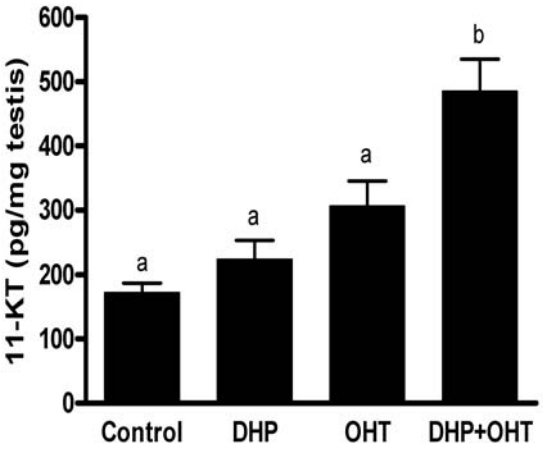

Figure 7 Release of 11-KT from zebrafish testicular explants**.** Amounts of 11-KT (mean ± SEM) released by zebrafish testis during overnight exposure to different conditions. Control, testis incubated without steroid; DHP, testis incubated with DHP (100 ng/ml); OHT; testis incubated with 11β-OHT (10 ng/ml); DHP+OHT, testis incubated with DHP (100 ng/ml) and 11β-OHT (10 ng/ml). Values represent compiled data from 2 independent experiments with 6 replicates per condition each. Bars marked with different letters are significantly different from each other (P<0.05).

DISCUSSION

In the present study, we cloned the open-reading frame of a zebrafish *pgr* cDNA, which encodes a protein of 617 amino acids. The N-terminal domain of the deduced zebrafish Pgr protein displayed low homology (7-24%; Supplemental Table 2) with PGRs from other vertebrate species. In contrast, the DNA-binding domain (DBD; 89-97%) and ligand-binding domain (LBD; 65-83%) are highly conserved between the zebrafish Pgr and other PGRs. The highly conserved DBD contains cysteine residues, constituting the two zinc finger motifs, as well as the P box (GSCKV) and D box (AGRND) sequences, which are important regions for the recognition of target gene sequences that are all conserved in the zebrafish Pgr. 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 [46], was not found in the zebrafish Pgr, so that a Pgr-mediated Mos/MAPK activation may not occur in zebrafish.

The result of our comparative analysis of Pgr amino acid sequences was congruent with the phylogenetic relationships among the major vertebrate clades [47]. The zebrafish Pgr formed a clade with other piscine Pgr proteins, while amphibian, reptilian and avian Pgr proteins, on the one hand, and mammalian PGRs, on the other, formed two separate clades. Our phylogenetic analysis is in accordance with the phylogenetic trees produced by other authors [48] prior to the characterization of the zebrafish Pgr.

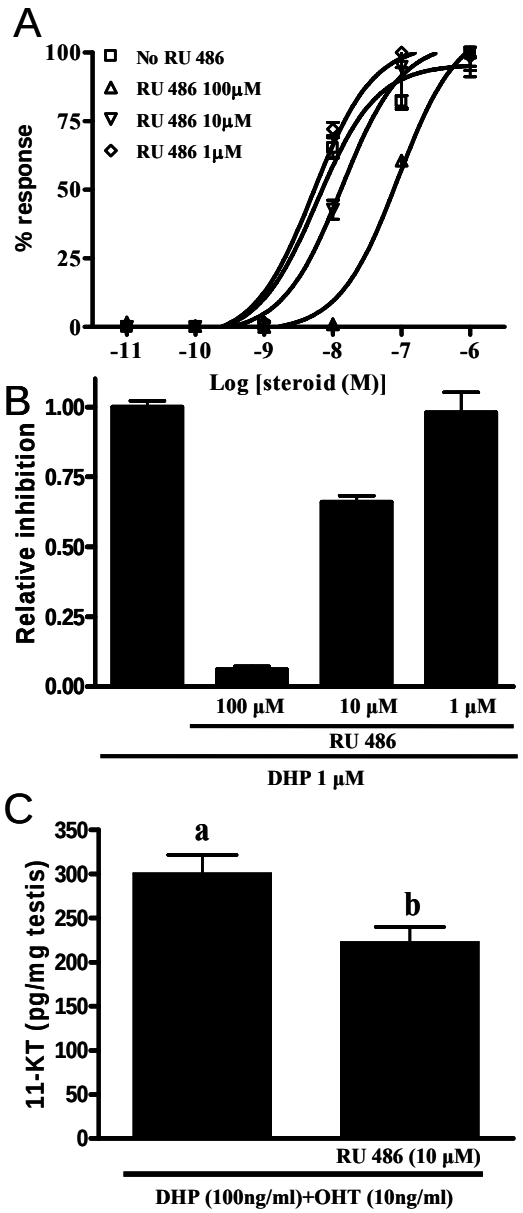

Figure 8 Effects of RU486 on DHP-stimulated 11-KT release. (A) Inhibition of DHP-induced, zebrafish Pgr-mediated transactivation of the MMTV promoter by RU486. The cells were incubated for 24 h with increasing concentrations of DHP (10 pM to 1 µM) with or without 1 µM, 10 µM or 100 µM RU486. Percentage (%) of response: values are given relative to the maximal amount of luciferase activity for each condition. (B) The cells were incubated for 24 h with fixed concentrations of DHP (1 µM) with or without 1 µM, 10 µM or 100 µM RU486, Data are expressed as the ratio of RU486:DHP. (C) Amounts of 11-KT released by zebrafish testis during overnight exposure to either DHP (100 ng/ml) plus 11*β*-OHT (10 ng/ml) or DHP (100 ng/ml) plus 11*β*-OHT (10 ng/ml) with RU486 (10 µM).

Data are expressed as mean ± SEM (n=6). Bars marked with different letters are significantly different from each other ($P<0.05$) (Student's t-test).

Two isoforms (forms A and B) encoded by the same gene but originating from different translational initiation at two in-phase ATG codons have been reported for chicken and human progesterone receptor homologues [49, 50]. In Japanese eel, *Anguilla japonica*, two distinct *pgr* genes have been reported [51, 52]. However, experimental trials to isolate additional *pgr* cDNAs or in silico approaches (e.g. searches of the *Danio rerio* ENSEMBL database [version 44.6e]; data not shown) to identify related sequences, did not provide evidence for the existence of additional *pgr*-like genes or mRNA isoforms from one gene in zebrafish.

We demonstrated that zebrafish Pgr is able to transactivate target genes in a progesterone-dependent manner. In the presence of DHP, zebrafish Pgr activated the transcription of a luciferase gene under control of the progesterone-regulated

MMTV-LTR promoter [53]. Moreover, transactivation was progesterone-specific, and DHP was the most effective steroid (EC_{50} = 8 nM). In mammals and chicken, P4 is considered to be a ligand for their PGRs. However, in teleost fish, DHP and/or 20*β*-S (the latter mainly for marine species) are the major progestins [54-56], and P4 is an intermediate in the synthesis of these steroids [57]. Our experiments showed that zebrafish testis tissue produced DHP in response to gonadotropic stimulation (see below). While no information is available on DHP plasma levels in zebrafish, 3-8 nM DHP was measured in blood plasma samples of spawning males in closely related fish species [58, 59]. Taken together, these results support the view that DHP is the native ligand for the zebrafish Pgr.

In adult zebrafish, *pgr* mRNA is expressed predominantly in testis but is detectable at low levels in other tissues, although it has a less broad expression pattern than the zebrafish androgen [35] or estrogen receptor [60] mRNAs. In mammals, PGRs were detected in uterus, ovary, vagina, testis, breast, brain, vascular endothelium, thymus, pancreatic islet, osteoblast-like cells, and lung [61]. In non-mammalian species, PGRs were also detected in testis and oviduct of chicken [49, 62], or oviduct and liver of turtle [63, 64]. In Japanese eel, *pgr2* mRNA was detected in gill, spleen, testis, brain, and ovary, whereas *pgr1* mRNA was observed in kidney, spleen, liver, and testis [52]. In a frog species, *pgr* mRNA has a broad expression pattern [65].

In zebrafish embryos, the *pgr* mRNA can not be detected at 0, 2 and 4 hpf; *pgr* mRNA is first detected at 8 hpf, and then *pgr* mRNA levels increase at 24 hpf. This shows that in zebrafish *pgr* mRNA is not maternally deposited in oocytes, but shows zygotic expression and may have a role during late embryonic development. In the mouse, there is little expression of *Pgr* mRNA until the blastocyst stage [66], and *Pgr* expression is not essential for embryonic viability [67].

During zebrafish gonad development, all individuals first develop an ovary containing oogonia and oocytes [25]. At approximately 3 wpf, this initial ovary either develops further into a mature ovary or starts transforming into a testis. At 4 wpf, the ovary contains numerous oocytes, while testes develop into 3 different types [37]. In this experiment, type I testes were used (i.e. thread-like gonads with low intensity of EGFP fluorescence) to represent males at 4 wpf [37]. Our results revealed that *pgr* and *piwil1* mRNA levels were higher in the developing ovary than in type I testis, while the meiosis marker *sycp3l* was found at similar levels in both sexes. Since germ cell proliferation starts earlier in females [25] and since Piwil1 protein is expressed in oogonia and early oocytes [38], the higher germ cell number in females may explain the higher level of both *piwil1* and *sycp3l* in ovaries, while the detection of *sycp3l* mRNA in testes may reflect the presence of residual, perhaps degenerating oocytes in the transforming testis (spermatocytes are still absent). At 8 wpf, the *pgr* mRNA levels had increased significantly in both sexes while the difference between sexes disappeared. At this age, meiosis had started in males [25]. Miura et al. showed that a function for DHP in male eel is to stimulate entry of germ cells into meiosis [13]. In Japanese eel [52] and chicken [62], *pgr* mRNA levels were also higher in testes of mature than of immature animals. We therefore speculate that first reaching (8 wpf) and then surpassing (12 wpf) female *pgr* mRNA expression levels may reflect the

entry of numerous germ cells into meiosis in the maturing testis; after all, there are many more spermatocytes than oocytes in (young) adult gonads. In ovarian tissue, however, *pgr* mRNA levels decreased significantly when the females developed towards young adults. This may be based on a dilution effect since ovarian tissue mass increased considerably in context with increases in oocyte growth due to vitellogenesis from 8 to 12 wpf, which is associated with stockpiling large amounts of maternal mRNAs in the oocytes [68], not including *pgr* mRNA, as we have shown in the present study.

We have no information on circulating DHP levels during gonad development in zebrafish while respective data are available from larger fish species. In male Japanese huchen, plasma DHP levels increased above detection limit with the appearance of meiotic cells in the testis [8]. In rainbow trout, a similar observation was made by Scott and Sumpter [7]. Dépêche and Sire [9] reported that rainbow trout testis tissue showed three periods of DHP production from 17*α*(OH)P4, in immature fish before the start of rapid spermatogonial proliferation, during the entry into meiosis, and in fully mature, spawning fish. Taken together, our data suggest that gonadal *pgr* mRNA expression patterns in zebrafish may be functionally related to the entry into meiosis, as has been demonstrated previously for Japanese eel [13]. The early presence of *pgr* mRNA levels in zebrafish testis and DHP production in immature rainbow trout males might indicate that additional functions are fulfilled during the initial stages of spermatogenesis, while there is already information available on the role for DHP during final maturation stages (e.g. composition of seminal fluid [10-12]; reproductive behaviour [14, 15]).

In boar testes, the PGR protein locates to type A and B spermatogonia [69], to primary spermatocytes and spermatids in rat [70], and to fully mature spermatozoa in dog [71]. In eel, *pgr1* mRNA was expressed in germ cells, Sertoli cells, and interstitial cells of testis, whereas *pgr2* mRNA was detected only in germ cells [13]. Human testicular PGR expression was found in some but not all germ cell types, in Sertoli cells and in Leydig cells in one study [72], while a much more restricted distribution to peritubular cells and to Leydig cells was reported in a study using four different antibodies and examining human and non-human primate testes [73]. In the present study, we found a strong in situ hybridization signal in the cytoplasm of Leydig cells and a weak staining in Sertoli cells. However, we found no evidence for *pgr* mRNA expression in germ cells, so that DHP effects on germ cells development are likely to be mediated by testicular somatic cells.

We have demonstrated that zebrafish testis tissue produced DHP in vitro when exposed to relatively high concentrations of rec-zfFsh, rec-zfLh or forskolin. In the steroidogenic pathways leading to androgens or DHP, 17*α*(OH)P4 holds a central position, as substrate for both 20β-hydroxysteroid dehydrogenase (catalyzing DHP production) and Cyp17a1 (catalyzing androgen production), while the production of 17*α*(OH)P4 depends on the StAR-mediated, gonadotropin-dependent, conversion of cholesterol to pregnenolone in the mitochondria. In salmonids and eel, it has been suggested that gonadotropin stimulates the testicular somatic cells to produce DHP precursor, probably 17*α*(OH)P4, which is then converted to DHP via the 20*β*-HSD

activity of spermatozoa [12, 57]. However, 20β-HSD activity is also present in immature rainbow trout testis when spermatozoa are still absent [74]. Ongoing work in our laboratory indicates that a significant stimulation of androgen release occurs already at 4- to 8-fold lower gonadotropin concentrations than an increase of DHP release (unpublished data). These results are compatible with the model that strong gonadotropic stimulation and hence high levels of the precursor 17α(OH)P4 are required to allow DHP production, while moderate gonadotropic stimulation would mainly result in androgen production.

In juvenile eel testis, DHP increases 11β-HSD activity, the enzyme catalyzing the final step in the production of the main androgen in fish, 11-KT [45]. Our results suggest that this stimulation occurs via a Prg-dependent manner also in adult zebrafish testis. On the other hand, androgens were shown to stimulate DHP production in Japanese eel [13], and to down-regulate Cyp17a activity in Japanese eel [13] and African catfish [75]. In the latter species, this down-regulation depended on the type of androgen and the stage of maturity; while testosterone shows down-regulatory effects in both immature and mature fish, 11-KT was only active in immature fish [76]. This leads to a model, in which androgen and progesterone production exert mutual control of their biosynthesis, provided that gonadotropic stimulation is sufficiently strong, possibly leading to a phased oscillation of DHP and androgen production.

In conclusion, we identified a progesterone receptor cDNA, exhibiting nuclear hormone receptor features, from zebrafish testis. The zebrafish progesterone receptor is expressed by Leydig and Sertoli cells, is best activated by its natural ligand (DHP) that is produced under strong gonadotropin stimulation, and may regulate germ cell differentiation (e.g. meiosis) and steroidogenesis.

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References

1. Clarke CL, Sutherland RL. Progestin regulation of cellular proliferation. Endocr Rev 1990; 11:266-301.
2. Schneider JS, Burgess C, Sleiter NC, DonCarlos LL, Lydon JP, O'Malley B, Levine JE. Enhanced sexual behaviors and androgen receptor immunoreactivity in the male progesterone receptor knockout mouse. Endocrinology 2005; 146:4340-4348.
3. Kamischke A, Nieschlag E. Progress towards hormonal male contraception. Trends Pharmacol Sci 2004; 25:49-57.
4. Calogero AE, Burrello N, Barone N, Palermo I, Grasso U, D'Agata R. Effects of progesterone on sperm function: mechanisms of action. Hum Reprod 2000; 15:28-45.
5. Lösel RM, Falkenstein E, Feuring M, Schultz A, Tillmann HC, Rossol-Haseroth K, Wehling M. Nongenomic steroid action: controversies, questions, and answers. Physiol Rev 2003; 83:965-1016.
6. Nagahama Y. 17*α*,20*β*-dihydroxy-4-pregnen-3-one, a maturation-inducing hormone in fish oocytes: Mechanisms of synthesis and action. Steroids 1997; 62:190-196.
7. Scott AP, Sumpter JP. Seasonal variations in testicular germ cell stages and in plasma concentrations of sex steroids in male rainbow trout (*Salmo gairdneri*) maturing at 2 years old. Gen Comp Endocrinol 1989; 73:46-58.
8. Amer MA, Miura T, Miura C, Yamauchi K. Involvement of sex steroid hormones in the early stages of spermatogenesis in Japanese huchen (*Hucho perryi*). Biol Reprod 2001; 65:1057-1066.
9. Dépêche J, Sire O. In vitro metabolism of progesterone and 17*α*-hydroxyprogesterone in the testis of the rainbow trout, *Salmo gairdneri* Rich., at different stages of spermatogenesis. Reprod Nutr Dev 1982; 22:427-438.
10. Ueda H, Kanbegawa A, Nagahama Y. Involvement of gonadotrophin and steroid hormones in spermiation in the amago salmon, *Oncorhynchus rhodurus*, and goldfish, *Carassius auratus*. Gen Comp Endocrinol 1985; 59:24-30.
11. Miura T, Yamauchi K, Takahashi H, Nagahama Y. Involvement of steroid hormones in gonadotropin-induced testicular maturation in male Japanese eel (*Anguilla japonica*). Biomed Res 1991; 12:241-248.
12. Miura T, Yamauchi K, Takahashi H, Nagahama Y. The role of hormones in the acquisition of sperm motility in salmonid fish. J Exp Zool 1992; 261:359-363.
13. Miura T, Higuchi M, Ozaki Y, Ohta T, Miura C. Progestin is an essential factor for the initiation of the meiosis in spermatogenetic cells of the eel. Proc Natl Acad Sci U S A 2006; 103:7333-7338.
14. Dulka JG, Stacey NE, Sorensen PW, van der Kraak GJ. A steroid sex pheromone synchronizes male-female spawning readiness in goldfish. Nature 1987; 325: 251-253.
15. Hong WS, Chen SX, Zhang QY, Zheng WY. Sex organ extracts and artificial hormonal compounds as sex pheromones to attract broodfish and to induce spawning of Chinese black sleeper (*Bostrichthys sinensis* Lacépède). Aquac Res

2006; 37: 529-534.
16. Pang Y, Dong J, Thomas P. Estrogen signaling characteristics of Atlantic croaker G protein-coupled receptor 30 (GPR30) and evidence it is involved in maintenance of oocyte meiotic arrest. Endocrinology 2008; 149:3410-3426.
17. Radu RA, Hu J, Peng J, Bok D, Mata NL, Travis GH. Retinal pigment epithelium-retinal G protein receptor-opsin mediates light-dependent translocation of all-trans-retinyl esters for synthesis of visual chromophore in retinal pigment epithelial cells. J Biol Chem 2008; 283:19730-19738.
18. Sugimoto Y, Narumiya S. Prostaglandin E receptors. J Biol Chem 2007; 282:11613-11617.
19. Thomas P. Characteristics of membrane progestin receptor alpha (mPRα) and progesterone membrane receptor component 1 (PGMRC1) and their roles in mediating rapid progestin actions. Front Neuroendocrinol 2008; 29:292-312.
20. Kolmakov NN, Kube M, Reinhardt R, Canario AV. Analysis of the goldfish *Carassius auratus* olfactory epithelium transcriptome reveals the presence of numerous non-olfactory GPCR and putative receptors for progestin pheromones. BMC Genomics 2008; 9:429.
21. Krietsch T, Fernandes MS, Kero J, Losel R, Heyens M, Lam EW-F, Huhtaniemi I, Brosens JJ, Gellersen B. Human homologs of the putative G protein-coupled membrane progestin receptors (mPRα, β, and γ) localize to the endoplasmic reticulum and are not activated by progesterone. Mol Endocrinol 2006; 20:3146–3164.
22. Briggs JP. The zebrafish: a new model organism for integrative physiology. Am J Physiol Regul Integr Comp Physiol 2002; 282:R3-R9.
23. McGonnell IM, Fowkes RC. Fishing for gene function-endocrine modelling in the zebrafish. J Endocrinol 2006; 189:425-439.
24. Santos EM, Workman VL, Paull GC, Filby AL, Van Look KJ, Kille P, Tyler CR. Molecular basis of sex and reproductive status in breeding zebrafish. Physiol Genomics 2007; 30:111-122.
25. Maack G, Segner H. Morphological development of the gonads in zebrafish. J Fish Biol 2003; 62:895-906.
26. Schulz RW, Bogerd J, Male R, Ball J, Fenske M, Olsen LC, Tyler CR. Estrogen-Induced alterations in *amh* and *dmrt1* expression signal for disruption in male sexual development in the zebrafish. Environ Sci Technol 2007; 41:6305-6310.
27. Van den Belt K, Wester PW, van der Ven LT, Verheyen R, Witters H. Effects of ethynylestradiol on the reproductive physiology in zebrafish (*Danio rerio*): time dependency and reversibility. Environ Toxicol Chem 2002; 21:767-775.
28. Leal MC, Cardoso ER, Nóbrega RH, Batlouni SR, Bogerd J, França LR, Schulz RW. Histological and stereological evaluation of zebrafish (*Danio rerio*) spermatogenesis with an emphasis on spermatogonial generations. Biol Reprod 2009; 81:177-187.
29. Krovel AV, Olsen LC. Expression of a *vas::EGFP* transgene in primordial germ cells of the zebrafish. Mech Dev 2002; 116:141-150.

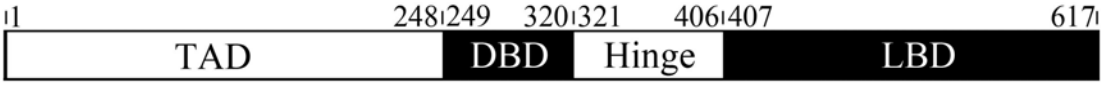
30. Westerfield M. The zebrafish book: a guide for the laboratory use of zebrafish (*Danio rerio*). 4th edn. Eugene: University of Oregon Press; 2000.
31. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol 1990; 215:403-410.
32. Higgins DG, Sharp PM. Fast and sensistive multiple sequence alignments on a microcomputer. Comput Appl Biosci 1989; 5:151-153.
33. Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 1987; 4:406-425.
34. Graham FL, van der Eb AJ. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology 1973; 52:456-467.
35. de Waal PP, Wang DS, Nijenhuis WA, Schulz RW, Bogerd J. Functional characterization and expression analysis of the androgen receptor in zebrafish (*Danio rerio*) testis. Reproduction 2008; 136:225-234.
36. Baulieu EE. On the mechanism of action of RU486. Ann NY Acad Sci 1991; 626:545-560.
37. Wang XG, Bartfai R, Sleptsova-Freidrich I, Orban L. The timing and extent of 'juvenile ovary' phase are highly variable during zebrafish testis differentiation. J Fish Biol 2007; 70:1-12.
38. Houwing S, Kamminga LM, Berezikov E, Cronembold D, Girard A, van den Elst H, Filippov DV, Blaser H, Raz E, Moens CB, Plasterk RHA, Hannon GJ, Draper BW, Ketting RF. A role for piwi and piRNAs in germ cell maintenance and transposon silencing in zebrafish. Cell 2007; 129:69-82.
39. Yano A, Suzuki K, Yoshizaki G. Flow-cytometric isolation of testicular germ cells from rainbow trout (*Oncorhynchus mykiss*) carrying the green fluorescent protein gene driven by trout *vasa* regulatory regions. Biol Reprod 2008; 78:151-158.
40. Bogerd J, Blomenrohr M, Andersson E, van der Putten HHAGM, Tensen CP, Vischer HF, Granneman JC, Janssen-Dommerholt C, Goos HJTh, Schulz RW. Discrepancy between molecular structure and ligand selectivity of a testicular follicle-stimulating hormone receptor of the African catfish (*Clarias gariepinus*). Biol Reprod 2001; 64:1633-1643.
41. Vischer HF, Teves AC, Ackermans JC, van Dijk W, Schulz RW, Bogerd J. Cloning and spatiotemporal expression of the follicle-stimulating hormone β subunit complementary DNA in the African catfish (*Clarias gariepinus*). Biol Reprod 2003; 8:1324-1332.
42. García-López A, Bogerd J, Granneman JCM, van Dijk W, Trant JM, Taranger GL, Schulz RW. Leydig cells express follicle-stimulating hormone receptors in African catfish. Endocrinology 2009; 150:357-365.
43. Leal MC, de Wall PP, García-López A, Chen SX, Bogerd J, Schulz RW. Zebrafish (*Danio rerio*) primary testis tissue culture: an approach to study testis function *ex vivo*. Gen Comp Endocrinol 2009; 162:134-138.
44. Schulz RW, van der Corput L, Janssen-Dommerholt C, Goos HJTh. Sexual steroids during puberty in male African catfish (*Clarias gariepinus*): serum levels and gonadotropin-stimulated testicular secretion in vitro. J Comp Physiol [B]

1994; 164:195-205.
45. Ozaki Y, Higuchi M, Miura C, Yamaguchi S, Tozawa Y, Miura T. Roles of 11*β*-hydroxysteroid dehydrogenase in fish spermatogenesis. Endocrinology 2006; 147:5139-5146.
46. Boonyaratanakornkit V, Scott MP, Ribon V, Sherman L, Anderson SM, Maller JL, Miller WT, Edwards DP. Progesterone receptor contains a proline-rich motif that directly interacts with SH3 domains and activates c-Src family of tyrosine kinases. Mol Cell 2001; 8:269-280.
47. Carroll RL. Vertebrate paleontology and evolution. New York: W. H. Freeman and Company; 1988.
48. Katsu Y, Bermudez DS, Braun E, Helbing C, Miyagawa S, Gunderson MP, Kohno S, Bryan TA, Guillette LJ, Iguchi T. Molecular cloning of the estrogen and progesterone receptors of the American alligator. Gen Comp Endocrinol 2004; 136:122-133.
49. Schrader WT, O'Malley BW. Progesterone-binding components of chick oviduct IV. characterization of purified subunits. J Biol Chem 1972; 247:51-59.
50. Kastner P, Krust A, Turcotte B Stropp U, Tora L, Gronemeyer H, Chambon P. Two distince estrogen-regulated promoters generate transcripts encoding the two functionally different human progesterone receptor forms A and B. EMBO J 1990; 9:1603-1614.
51. Todo T, Ikeuchi T, Kobayashi T, Kajiura-Kobayashi H, Suzuki K, Yoshikuni M, Yamauchi K, Nagahama Y. Characterization of a testicular 17*α*,20*β*-dihydroxy-4-pregnen-3-one (a spermiation-inducing steroid in fish) receptor from a teleost, Japanese eel (*Anguilla japonica*). FEBS Lett 2000; 465:12-17.
52. Ikeuchi T, Todo T, Kobayashi T, Nagahama Y. A novel progesterone receptor subtype in the Japanese eel, *Anguilla japonica*. FEBS Lett 2002; 510:77-82.
53. Truss M, Beato M. Steroid hormone receptors: interaction with deoxyribonucleic acid and transcription factors. Endocr Rev 1993; 14:459-479.
54. Barry TP, Santos AJ, Furukawa K, Aida K, Hanyu I. Steroid profiles during spawning in male common carp. Gen Comp Endocrinol 1990; 80:223-231.
55. Thomas P. Trant M. Evidence that 17*α*,20*β*,21-trihydroxy-4-pregnen-3-one is a maturation-inducing steroid in spotted seatrout. Fish Physiol Biochem 1989; 7:185-191.
56. King W 5th, Ghosh S, Thomas P, Sullivan CV. A receptor for the oocyte maturation-inducing hormone 17*α*,20*β*,21-trihydroxy-4-pregnen-3-one on ovarian membranes of striped bass. Biol Reprod 1997; 56:266-271
57. Nagahama Y. Endocrine regulation of gametogenesis in fish. Int J Dev Biol 1994; 38:217–229.
58. Kobayashi M, Aida K, Hanyu I. Gonadotropin surge during spawning in male goldfish. Gen Comp Endocrinol 1986; 62:70-79.
59. Barry TP, Santos AJG, Furukawa K, Aida K, Hanyu I. Steroid profiles during spawning in male common carp. Gen Comp Endocrinol 1990; 80:223-231.
60. Menuet A, Pellegrini E, Anglade I, Blaise O, Laudet V, Kah O, Pakdel F.

Molecular characterization of three estrogen receptor forms in zebrafish: binding characteristics, transactivation properties, and tissue distributions. Biol Reprod 2002; 66:1881-1892.
61. Graham JD, Clarke CL. Physiological action of progesterone in target tissues. Endocr Rev 1997; 18:502-519.
62. González-Morán MG, Guerra-Araiza C, Campos MG, Camacho-Arroyo I. Histological and sex steroid hormone receptor changes in testes of immature, mature, and aged chickens. Domest Anim Endocrinol 2008; 35:371-379.
63. Reese JC, Callard IP. Two progesterone receptors in the oviduct of the freshwater turtle *Chrysemys picta*: Possible homology to mammalian and avian progesterone receptor systems. J Steroid Biochem 1989; 33:297-310.
64. Riley D, Reese JC, Callard IP. Hepatic progesterone receptors: Characterization in the turtle *Chrysemys picta*. Endocrinology 1988; 123:1195-1201.
65. Wang L, Sanyal S, Oh DY, Kim J, Ju JW, Song K, Kim JW, Kwon HB, Choi H. Molecular cloning and characterization of an amphibian progesterone receptor from *Rana dybowskii*. Gen Comp Endocrinol 2004; 135:142-149.
66. Hou Q, Gorski J. Estrogen receptor and progesterone receptor genes are expressed differentially in mouse embryos during preimplantation development. Proc Natl Acad Sci U S A 1993; 90:9460-9464.
67. Conneely OM, Mulac-Jericevic B, Lydon JP, De Mayo FJ. Reproductive functions of the progesterone receptor isoforms: lessons from knock-out mice. Mol Cell Endocrinol 2001; 179:97-103.
68. Pelegri F. Maternal factors in zebrafish. Dev Dyn 2003; 228:535-554.
69. Kohler C, Riesenbeck A, Hoffmann B. Age-dependent expression and localization of the progesterone receptor in the boar testis. Reprod Domest Anim 2007; 42:1-5.
70. Galena HJ, Pillai AK, Terner C. Progesterone and androgen receptors in non-flagellate germ cells of the rat testis. J Endocrinol 1974; 63:223-237.
71. Sirivaidyapong S, Bevers MM, Gadella BM, Colenbrander B. Induction of the acrosome reaction in dog sperm cells is dependent on epididymal maturation: the generation of a functional progesterone receptor is involved. Mol Reprod Dev 2001; 58:451-459.
72. Shah C, Modi D, Sachdeva G, Gadkar S, Puri C. Coexistence of intracellular and membrane-bound progesterone receptors in human testis. J Clin Endocrinol Metab 2005; 90:474-483.
73. Luetjens CM, Didolkar A, Kliesch S, Paulus W, Jeibmann A, Bocker W, Nieschlag E, Simoni M. Tissue expression of the nuclear progesterone receptor in male non-human primates and men. J Endocrinol 2006; 189:529-539.
74. Vizziano D, LeGac F. Effect of gonadotropin type II and 17-hydroxy-4-pregnene-3,20-dione on 17,20β-dihydroxy-4-pregnen-3-one production by rainbow trout testes at different developmental stages. Fish Physiol Biochem 1998; 19:269-277.
75. Cavaco JEB, Blijswijk Van B, Leatherland JF, Goos HJT, Schulz RW. Androgen-induced changes in Leydig cell ultrastructure and steroidogenesis in juvenile African catfish, *Clarias gariepinus*. Cell Tissue Res 1999; 297:291-299.

76. Schulz RW, Liemburg M, Garcia-Lopez A, Dijk W, Bogerd J. Androgens modulate testicular androgen production in African catfish (*Clarias gariepinus*) depending on the stage of maturity and type of androgen. Gen Comp Endocrinol 2008; 156:154-163.

Supplemental information



Supplemental Figure 1 Specific domains of the zebrafish progesterone receptor
Schematic representation of the zebrafish Pgr and the localization of specific domains: TAD, transactivation domain; DBD, DNA-binding domain; Hinge, hinge domain; LBD, ligand-binding domain. Previously, the TAD, DBD, hinge and LBD domains were named A/B, C, D and E/F domains, respectively. The numbers above each box refer to the amino acid positions in each domain.

Supplemental Table 1 Primers used in real-time, quantitative PCR.

Target	GenBank accession no.	Primer*	Sequence (5'→3')*
pgr	FJ409244	2901 (Fw1)	GGATTGTCAGATGGTCCAAATCTC
		2902 (Rv1)	GCCCATCCAGGAATACTGAATTAGT
		2913 (Fw2)**	GGGCCACTCATGTCTCGTCTA
		2914 (Rv2) **	TCTCCACTCTGAAAATATGTGGACTTT
piwil1	NM_183338	2542 (Fw)	GATACCGCTGCTGGAAAAAGG
		2543 (Rv)	GCAAGACACACTTGGAGAACCA
sycp3l	BC115343	2730 (Fw)	AGAAGCTGACCCAAGATCATTCC
		2731 (Rv)	AGCTTCAGTTGCTGGCGAAA
actb1	BC045846	2647 (Fw)**	TGCTCTGTATGGCGCATTGA
		2648 (Rv) **	GCTCCTCCCCCTGTTAGACAAC

*Sequences are given for the forward (Fw) and reverse (Rv) primers.

**Primers used in combination with cDNA from laser-microdissected samples.

Supplemental Table 2 Percentage amino acid identity of the zebrafish Pgr compared with PGRs from other species.

	Full length	A/B	DBD	Hinge	LBD
Eel Pgr1	57.1	23.9	97.2	57.0	83.2
Eel Pgr2	55.9	21.1	91.7	64.0	81.1
Human PGR	43.6	13.8	90.3	36.0	67.4
Dog PGR	44.0	12.2	90.3	37.2	66.8
Chicken PGR	47.1	12.1	90.3	44.7	66.3
Frog PGR	48.0	14.5	88.9	45.3	68.9
Mouse PGR	44.2	13.9	90.3	38.4	65.3
Rat PGR	43.9	11.5	90.3	38.4	66.3
Korean wild frog PGR	46.4	12.1	83.3	41.5	67.4
Rabbit PGR	44.0	13.0	90.3	36.6	66.8
Sheep PGR	62.9	7.1	90.3	37.2	66.8
Bovine PGR	57.6	14.1	90.3	38.4	66.8
Freshwater turtle PGR	45.8	13.9	90.3	46.5	66.8

The amino acid sequences of the different PGRs were gathered from the GenBank database: Japanese eel (*Anguilla japonica*) (receptor 1, BAA89539; receptor 2, AB028024), human (*Homo sapiens*; M15716), rabbit (*Oryctolagus cuniculus*; M14547), bovine (*Bos taurus*; AY656812), dog (*Canis lupus familiaris*; AF177470), sheep (*Ovis aries*; Z66555), rat (*Rattus norvegicus*; L16922), mouse (*Mus musculus*; M68915), frog (*Xenopus laevis*; AF279335), chicken (*Gallus gallus*; P07812), Korean wild frog (*Rana dybowskii*; AF431813), freshwater turtle (*Pseudemys nelsoni*; AB301062). Domain by domain homology analysis with the zebrafish Pgr was performed using MegAlign/DNASTAR software. See Figure 1 for key to the specific domains nomenclature.

Chapter 3

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Progesterone stimulates early stages of spermatogenesis in zebrafish

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Abstract
Recently, evidence has been provided for multiple regulatory functions of progestins during the late mitotic and meiotic phases of spermatogenesis in teleost fish. Our previous studies suggested that 17*α*,20*β*-dihydroxy-4-pregnen-3-one (DHP), potentially via Sertoli cells that express progesterone receptor (Pgr)-encoding mRNA, can contribute to the regulation of zebrafish spermatogenesis. To further our understanding of the function of progestin at early spermatogenetic stages, we investigated in the present study the expression of genes reflecting Sertoli cell function and spermatogenic development in adult zebrafish testis after DHP treatment. Using estrogen-mediated down-regulation of androgen production to interrupt spermatogenesis, we first studied the effect of exposure to DHP *in vivo*. After DHP treatment, all differentiating germ cell types were abundantly present and analysis of a DNA-synthesis marker (BrdU) showed high activity in testis tissue of DHP-treated animals. Besides, transcripts of two Sertoli cell-derived genes *anti-Müllerian hormone* (*amh*) and *gonadal soma-derived growth factor* (*gsdf*) were up-regulated. Moreover, three other genes of the insulin-like growth factor signalling system, *insulin-like growth factor 2b* (*igf2b*), *insulin-like growth factor 3* (*igf3*) and *insulin-like growth factor 1b receptor* (*igf1rb*), were up-regulated as well. We further analyzed the relationship between these genes and DHP treatment, using a recently developed, primary zebrafish testis tissue culture system. In the presence of DHP, only *igf1rb* mRNA levels showed a significant increase. Taken together, our results show that DHP treatment induces the proliferation of early spermatogonia, their differentiation into late spermatogonia and spermatocytes, as well as gene expression of markers for these germ cell stages. Moreover, our data suggest that DHP-stimulated spermatogenesis involves up-regulation of the activity of the Igf signalling system.

Introduction

In teleost fish, progestins 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), are known to regulate sperm maturation and hydration (Ueda *et al*. 1985; Miura *et al*. 1992). In addition, DHP induced DNA synthesis and the initiation of meiosis in an organ culture system for juvenile Japanese eel (*Anguilla japonica*) testis explants (Miura *et al*. 2006). Using the same experimental set-up, Miura and colleagues have moreover reported that DHP induced 11*β*-hydroxysteroid dehydrogenase (short form) activity, the enzyme catalyzing the final step in the production of the main androgen in fish, 11-KT (Ozaki *et al*. 2006). More recently, trypsinogen expression in the Sertoli cells surrounding spermatogonia was shown to be a critical element of the molecular mechanism of DHP-stimulated entry into meiosis in eel (Miura *et al*. 2009).

There are two types of progesterone receptors in fish. The classical nuclear progesterone receptor (Pgr) functions primarily as ligand-dependent transcription factor to regulate target gene expression (Conneely *et al*. 2002), while membrane-associated progesterone receptors are involved in nongenomic mechanism

(Thomas 2008). While both types of progesterone receptors types are highly expressed in fish testis (Ikeuchi *et al*. 2002; Hanna and Zhu 2009), the membrane-associated progesterone receptor is restricted to germ cells (Hanna and Zhu 2009, Thomas *et al*. 2005), in contrast to a broader expression pattern of the nuclear Pgr forms. In Japanese eel, *pgr1* mRNA was expressed in germ cells, Sertoli cells, and interstitial cells of testis, whereas *pgr2* mRNA was detected only in germ cells by using RT-PCR (Miura *et al*. 2006). In zebrafish (*Danio rerio*), showing only one type of Pgr, we found *pgr* mRNA expression in Leydig and Sertoli cells (Chen *et al*. 2010). In Atlantic salmon (*Salmo salar* L.) the *in situ* hybridization signal for *pgr* mRNA was restricted to Sertoli cells surrounding spermatogonia in testes at the onset of spermatogenesis (Chapter 3). Functionally, our previous work indicated that in adult zebrafish, DHP induced 11*β*-Hsd activity in a Pgr-dependent manner (Chen *et al*. 2010). Moreover, we have observed an increase in testicular *pgr* mRNA expression in association with the appearance of late type B spermatogonia in Atlantic salmon (Chapter 3). Since male germ cell development depends primarily on their interaction with the somatic Sertoli cells, it is reasonable to assume that Sertoli cell-derived factors mediate DHP-regulated steps in spermatogenesis in fish as was shown in the case of trypsinogen expression (see above).

To further understand the function of DHP during early stages of spermatogenesis in zebrafish, we have used an *in vivo* experimental model that is based on estrogen-induced interruption of spermatogenesis. This approach and a primary zebrafish testis tissue *ex vivo* culture system (Leal *et al*. 2009) were used in the present study to investigate the effects of DHP on spermatogenesis *in vivo* and *ex vivo*, respectively, including determination of the expression of genes reflecting aspects of Sertoli cell function and spermatogenic development in adult zebrafish testis after DHP treatment in order to identify potential factors mediating DHP action.

Material and methods

Fish stocks

Adult (>90 dpf) male outbred zebrafish were used for experimental purposes in the current study. Animal culture, performed using standard conditions for this species (Westerfield 2000), handling and experimentation were consistent with the Dutch national regulations, and were approved by the Life Science Faculties Committee for Animal Care and Use in Utrecht (The Netherlands).

In vivo exposure to sex steroids

In order to investigate DHP-induced spermatogenesis *in vivo*, zebrafish were subjected to a two-step treatment. First, the fish were kept for a period of 3 weeks in water containing 10 nM E_2 (Sigma-Aldrich, Zwijndrecht, The Netherlands). This regime down-regulated testicular androgen production and interrupted spermatogenesis by inhibiting the proliferation of type A spermatogonia and their differentiation into type B spermatogonia (de Waal *et al*. 2009). For the second step,

exposure to 10 nM E_2 was either continued for another two weeks (control group), or DHP was added to the water, so that during the final two weeks the experimental group was kept in water containing 10 nM E_2 and 100 nM DHP.

As described by de Waal *et al*. (2009), exposure was performed in a semi-static system in 13 L glass tanks at a temperature of 27°C. Fish were transferred to the experimental tanks 48 h before initiating exposure. The water was refreshed daily by moving the fish to a second set of identically prepared tanks. Both 10 mM E_2 and 100 mM DHP stock solution were prepared in deionized water separately by sonication, and then further diluted in aquarium water. During the last 6 h of exposure, 5-bromo-2-deoxyuridine (BrdU, a DNA synthesis/proliferation marker) (Sigma-Aldrich) was added at a concentration of 3 mg/ml to all aquaria.

Fish were euthanized in ice water and total body weight was measured. Both testes of each animal were excised, weighed and the gonadosomatic index (GSI; *i.e.* the ratio between testis weight and body weight) was calculated. One testis was snap frozen in liquid nitrogen and stored at -80°C until RNA extraction for gene expression analysis. The other testis was used for acute *ex vivo* steroid release bioassays, and then processed for morphological analysis or immunocytochemistry, as described previously (Leal *et al*. 2009).

***Ex vivo* exposure**

To determine if DHP directly regulates the expression of Sertoli cell-derived factors that showed a change in expression after DHP treatment *in vivo*, an *ex vivo* organ culture system for adult zebrafish testis explants was used, as described recently by Leal *et al*. (2009). In this system, the basal medium is free of growth factors, and the testicular steroidogenic capacity is down-regulated spontaneously during the first 48 hours, so that there is little interference from endogenously produced steroids. For the *ex vivo* experiment, adult fish were first kept in water containing 10 nM E_2 for a period of 3 weeks. For each individual, one testis served as control for the contralateral one, such that one testis was incubated under control conditions (basal medium only), while the other one was incubated in the presence of 200 nM DHP. Incubations took place in a humidified air atmosphere at 25ºC for 7 days, and medium was refreshed once after three or four days. After incubation, testicular tissue explants were snap frozen in liquid nitrogen and stored at -80°C until RNA extraction for gene expression analysis.

Acute basal *ex vivo* steroid secretion by zebrafish testicular explants

The acute *ex vivo* steroid release bioassay, was first described for African catfish (Schulz *et al*. 1994) and recently has been adapted for zebrafish testis (de Waal *et al*. 2009). Incubations lasted 6 h in a humidified air atmosphere at 25ºC in 96-well flat-bottom plates (Corning Inc., New York, USA) using a final volume of 200 µl. Basal culture medium consisted of 15 g/L Leibovitz L-15 (Invitrogen) supplemented with 10 mM Hepes (Merck), 0.5% w/v bovine serum albumin fraction V (Roche, Mannheim, Germany), 0.4 mg/L amphotericine B (Fungizone®; Invitrogen) and 200,000 U/L penicillin/streptomycin (Invitrogen); pH was adjusted to 7.4. Testis

tissue was incubated in basal medium to study basal androgen release. Testis tissue was sampled from untreated adults, and from fish either exposed to 10 nM E_2, or from fish exposed to 10 nM E_2 and 100 nM DHP *in vivo*, as described above. After 6 hrs of incubation, the medium was harvested and used for quantification of 11-KT (n = 8~10 per condition). The experiment served to study if the previously reported, DHP-induced stimulation of testicular 11β-hydroxysteroid dehydrogenase (11βHsd) would be reflected in significant differences of basal androgen release.

Immunocytochemistry

After *ex vivo* incubation, testicular explants were fixed, embedded, sectioned, and then processed for BrdU-immunodetection, as described previously (Leal *et al*. 2009). The detection of Piwil1 protein by immunocotychemistry has been carried out as described previously by Houwing *et al*. (2007), using 5 µm paraffin sections from adult zebrafish testis after fixation in 4% phosphate-buffered paraformaldehyde.

***In situ* hybridization**

Localization of *gonadal soma-derived growth factor* (*gsdf*) mRNA by *in situ* hybridization was done as described previously (Chen *et al*. 2010), using 10 µm cryo-sections prepared from 4% w/v paraformaldehyde-fixed testis tissue from sexually mature zebrafish testis. The localization of *daz-like gene* (*dazl*) mRNA was done in a similar way as *gsdf* mRNA, except that testis tissue was dehydrated and embedded in paraffin, according to conventional techniques, before 5 µm thick sections were used for hybridization with the antisense *dazl* cRNA probe. Neighboring sections were stained with hematoxylin-eosin to distinguish the type of germ cells showing *in situ* hybridization signal. Specific primers for *gsdf* (2643, 5'-GGGCGGGTGTTATTAACCCTCACTAAAGGGCTGGAGCATCTGCGGGAGT CATTGAA-3', and 2685, 5'-CCGGGGGGTGTAATACGACTCACTATAGGGCCA GTGATGCTGAACTACGGCTAGTTTGTGTT-3') were designed to PCR-amplify 560 bp cDNA fragments for sense and antisense digoxigenin-labeled cRNA probe synthesis, according to Vischer *et al*. (2003). Sense and antisense *dazl* cRNA probes were generated as described in Bontems *et al*. (2009).

Gene expression analysis

For gene expression analysis, total RNA was extracted from testicular samples using RNAqueous®-Micro Kit (Ambion, Austin, TX, USA), following the manufacturer's instructions. Synthesis of cDNA from total RNA was performed as described previously (Chen *et al*. 2010). Specific primers for zebrafish *steroidogenic acute regulatory protein* (*star*), *piwi-like 1 (Drosophila)* (*piwil1*), *dazl*, *synaptonemal complex protein 3 like* (*sycp3l*), *gsdf*, *insulin-like growth factor 1* (*igf1*), *insulin-like growth factor 2b* (*igf2b*), *insulin-like growth factor 3* (*igf3*), *insulin-like growth factor 1 receptors* (*igf1ra* and *igf1rb*) and one internal control gene *bactin1* were designed for SYBR green expression analysis (Applied Biosystems). Specific primers and FAM-labelled probes for *amh* and the two endogenous controls (*i.e. 18S ribosomal RNA* [*18s rRNA*] and *elongation factor 1α* [*ef1α*]) were designed for TaqMan

expression analysis. The specificity and amplification efficiency of each primer combination was confirmed on serial dilutions of testis cDNA (results not shown). Respective primer and probe sequences are shown in Supplemental Table 1. All real-time, quantitative PCRs (qPCR) were performed in 20 µl reactions and C_t values determined in a 7900HT Real-Time PCR System (Applied Biosystems) using default settings. Before normalizing expression data, we analysed the stability of the internal control genes from *in vivo* and *ex vivo* experiments. *In vivo*, only *18s rRNA* showed stable expression between experimental groups. *Ex vivo*, all three internal control genes showed stable expression between experimental groups. Therefore, *18s rRNA* was used to normalize data from the *in vivo* experiment, while the geometric mean (Vandesompele *et al*. 2002) of the three control genes was used to normalize the *ex vivo* data (supplemental Fig. 1). The comparative C_t method was used to calculate the relative mRNA levels (Schmittgen and Livak 2008).

Statistical analysis

For the *in vivo* exposure experiment, differences between treatment groups for the measured parameters, (*i.e.* relative mRNA level, GSI) were compared by Student's t test. For the *ex vivo* gene expression experiments, differences between treatment groups were tested for statistical significance using the paired t-test. The analyses were performed using GraphPad Prism4 software package (GraphPad Software, San Diego, CA). Data are presented as the mean ± SEM.

Results

Localization of Piwil1 protein and of *gsdf* and *dazl* mRNA in zebrafish testis

In order to evaluate the suitability as germ cell markers, we localized the three genes in adult testis tissue. The strongest signal for Piwil1 protein was found in the cytoplasm of type A spermatogonia (Fig. 1A, B), in single cells, pairs, and cysts of 4 type A spermatogonia. In later spermatogonial generations, the labelling was very weak. No labelling was found in meiotic or post-meiotic germ cells.

A clear *in situ* hybridization signal for *dazl* mRNA was detected in all spermatogonial generations, while *dazl* mRNA was absent from spermatocytes, spermatids, and spermatozoa (Fig. 1C, D). The staining appeared to be more intense in larger spermatogonial cyst (Fig. 1C), and was then abruptly down-regulated for the remainder of spermatogenesis.

In contrast to the two previous proteins/genes, *gsdf* mRNA was not expressed in germ cells but was found scattered throughout the testis in this cytoplasmic extensions that surrounded groups of germ cells (Fig. 1E). Higher magnifications showed that triangular areas often close to the tubular wall were also stained intensely (Fig. 1F). This staining pattern corresponds to the thin cytoplasmic extensions of Sertoli cells and their perinuclear cytoplasmic areas, respectively. There were no differences in the staining intensity between Sertoli cells contacting germ cells at different stages of the spermatogenesis.

The levels of expression of Piwil1 protein, *dazl*, and *sycp3l* mRNAs are

summarised schematically in relation to the stage of spermatogenesis in supplemental figure 2.

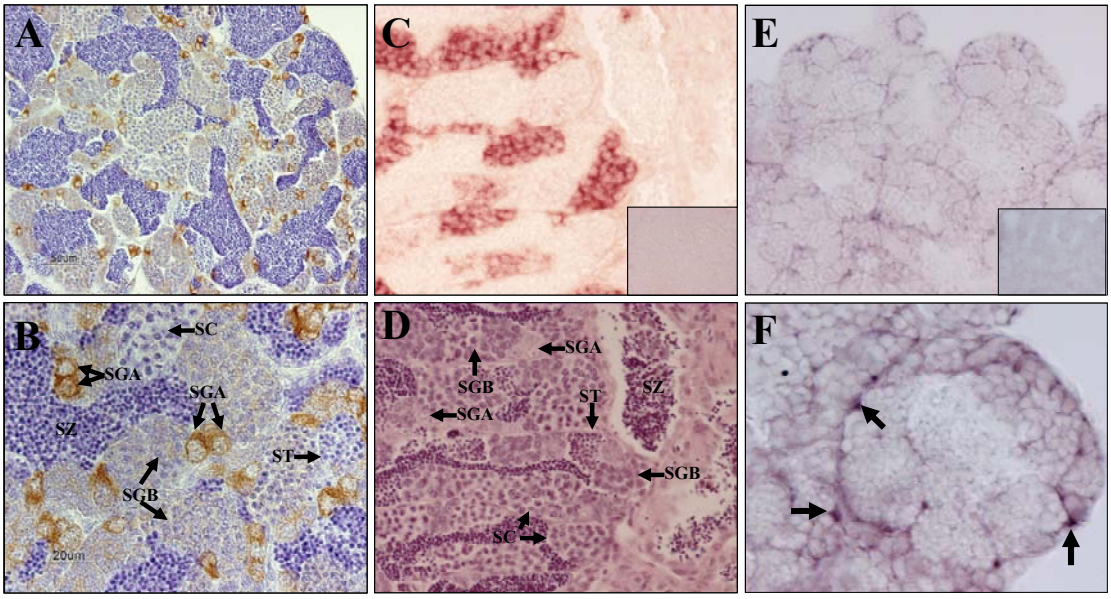

Figure 1 Immunohistochemistry analyses of Piwil1, and *In situ* hybridization analyses of *gsdf* mRNA and *dazl* mRNA in sexually mature zebrafish testis. Low (A) and high (B) magnification of immunostaining of Piwil1 of a paraffin-section showed strong staining in cytoplasm of type A spermatogonia, and very weak staining in type B spermatogonia. Note that spermatocytes, spermatid, and spermatozoa remained unstained. Consecutive paraffin-sections, hybridized with the *dazl* antisense cRNA probe (C), or stained with hematoxylin-eosin (D) showed strong staining in cytoplasm of type B spermatogonia, and weak staining in type A spermatogonia. Note that spermatocytes, spermatid, and spermatozoa remained unstained. Insert in (C) shows that the sense cRNA probe did not result in specific staining. Low (E) and high (F) magnification of a cryosection, hybridized with the *gsdf* antisense cRNA probe showed signal in the spermatogonial cysts at different developmental stage. Inset in (E) shows that the sense cRNA probe did not result in specific staining. Black block arrows in (F) indicates Sertoli cells cytoplasm. SGA, type A spermatogonia; SGB, type B spermatogonia; SC, spermatocytes; ST, spermatid; SZ, spermatozoa.

In vivo effects of DHP on zebrafish testis

The GSI of zebrafish exposed to DHP was twice as high as in the control group (Fig. 2A, $P<0.01$). Qualitative morphological analysis of testis samples collected from zebrafish under control conditions (*i.e.* exposed to E_2 for 5 weeks) showed that the testes were depleted of differentiating germ cell types (*i.e.* type B spermatogonia, spermatocytes and spermatids) (Fig. 2C, D) usually present in the testis of untreated adults (supplemental Fig. 3). Testis samples collected following 2 weeks of DHP exposure, on the other hand, showed all these types of differentiating germ cells in abundant numbers (Fig. 2E, F).

To further confirm that DHP treatment stimulated germ cell proliferation and differentiation *in vivo*, we monitored BrdU incorporation. While BrdU-labelling was found in only a limited number of spermatogonia in testis under control conditions

(Fig. 2C, D), BrdU-labelled germ cells were abundantly present in testis of DHP-treated fish (Fig. 2E, F). A BrdU-labelling was founding type A and type B spermatgonia as well as in primary spermatocytes, *i.e.* in all germ cell types theoretically able to incorporate BrdU during the short-term exposure of 6 hrs.

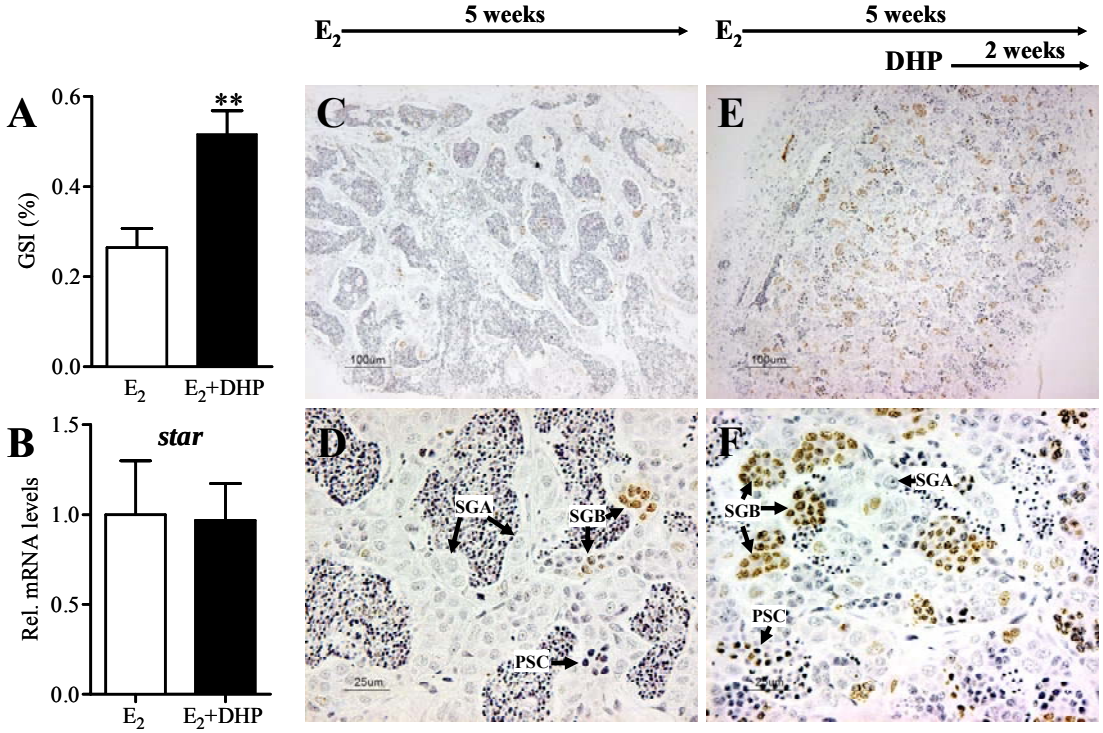

Figure 2 DHP treatment *in vivo* reversed the inhibitory effects of E_2 pretreatment on adult zebrafish spermatogenesis. (A) Gonado-somatic index (GSI) of zebrafish maintained in the absence or the presence of 100 nM DHP during last 2 weeks of total 5 weeks E_2 (10 nM) *in vivo* exposure. (B) Testicular relative (Rel.) mRNA levels of *star* of zebrafish maintained in the presence of 100 nM DHP during last 2 weeks of total 5 weeks E_2 (10 nM) *in vivo* exposure. Bars represent the mean (± SE) relative mRNA levels, normalized to internal control gene (*18s rRNA*). (C-F) BrdU labelling of testis tissue from zebrafish maintained in the presence of 10 nM E_2 for 5 weeks *in vivo* exposure (C, D), or also exposed to 100 nM DHP (E, F) during the last 2 weeks. For both exposure groups, BrdU was present during the last 6 h of exposure. Note that BrdU-labelled germ cells are much more abundant in DHP-treated testis. Bars marked with ** are significantly different (P<0.01, student's t-test) from their respective controls.

In order to examine the possibility that DHP exposure may have, at least in part, re-activated the E_2-inhibited steroidogenic system, we quantified the mRNA level of *star*, which controls the rate-limiting step in gonadotropin-stimulated steroidogenesis (*i.e.* the transfer of cholesterol from the outer to the inner mitochondrial membrane); we also quantified the amount of 11-ketotestosterone (11-KT) released from testis tissue. There was no difference in *star* mRNA levels between control and DHP-treated fish (Fig. 2B). While the basal release of 11-KT from testes of untreated controls usually is 50-100 pg 11-KT/mg tissue and hence clearly (~5-fold) above the limit of detection (García-López *et al*. 2010), no 11-KT was detected in medium harvested

from testes of males exposed to E_2 alone or in combination with DHP. We conclude that DHP treatment did not modulate the E_2-induced inhibition of testicular androgen production that has been observed previously (de Waal *et al*. 2009).

Data from gene expression analysis indicated that the morphological observations (see above) were in accordance with significantly elevated expression levels of all three germ cells markers used: *piwil1* (~2 fold), *dazl* (~2 fold), and *sycp3l* (~4 fold) in testis of DHP-treated fish compared to the control group (Fig. 3A); these three genes represent type A spermatogonia, all spermatogonia, and spermatocytes, respectively. Moreover, the mRNA expression levels of the Sertoli cell-specific genes *gsdf* and *amh,* and insulin-like growth factor signalling related genes *igf2b*, *igf3* and *igf1rb*, were all significantly increased in testis from DHP-treated zebrafish (Fig. 3A).

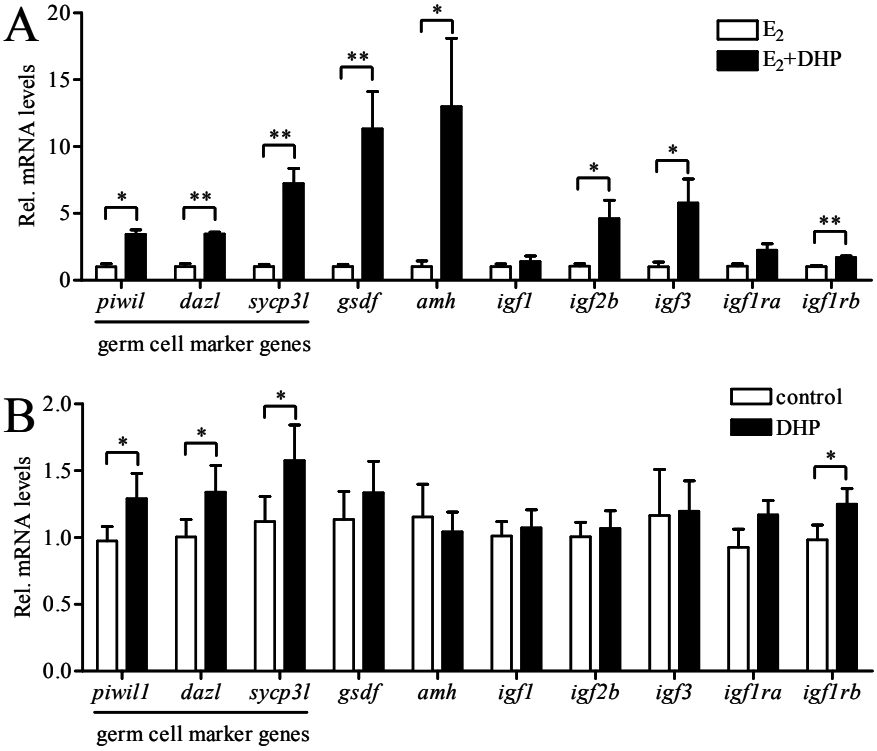

Figure 3 DHP regulation of genes representative and involved in spermatogenesis.

(A) Testicular relative (Rel.) mRNA levels of germ cell mark genes (*piwil1*, *dazl* and *sycp3l*), the Sertoli cell-specific genes (*gsdf*, *amh*), and insulin-like growth factor signalling system related genes (*igf1*, *igf2b*, *igf3*, *igf1ra*, *igf1rb*) of zebrafish maintained in the presence of 100 nM DHP during last 2 weeks of total 5 weeks E_2 (10 nM) *in vivo* exposure. Bars represent the mean (± SEM; n =5) relative mRNA levels, normalized to internal control gene (*18s rRNA*). Bars marked with * (P<0.05), ** (P<0.01) are significantly different (student's t-test) from their respective controls.

(B) Relative (Rel.) mRNA levels of germ cell mark genes (*piwil1*, *dazl*, *sycp3l*), the Sertoli cell-specific genes (*gsdf*, *amh*), and insulin-like growth factor signalling system related genes (*igf1*, *igf2b*, *igf3*, *igf1ra*, *igf1rb*) of testicular explants treated with 200 nM DHP *ex vivo* for 7 days, following donor adult zebrafish kept in water containing 10 nM E_2 for a period of 3 weeks. Bars represent the mean (± SEM; n = 9) relative mRNA levels, normalized to geometric mean of three internal control genes (*18s rRNA, ef1α* and *bactin1*). Bars marked with * (P<0.05) are significantly different (paired t-test) from their respective controls.

Effect of DHP on zebrafish testicular physiology *ex vivo*

The limited tissue mass after *ex vivo* culture, does not allow carrying out gene expression as well as morphological analysis on the same samples. We decided to analyse testicular explants for gene expression, since the results from our *in vivo* experiments indicated that data on the expression of markers by spermatogonia and spermatocytes were in accordance with morphological observations (*i.e.* both reflected increased mass and proliferation activity of spermatogonia and spermatocytes). After 7 days *ex vivo* DHP treatment, testicular explants showed significantly increased expression levels of germ cell gene markers, *i.e. piwil1*, *dazl* and *sycp3l*, compared with control (Fig. 3B, $P<0.05$). Next to germ cell marker genes, only *igf1rb* expression levels showing significantly increase compared with controls (Fig. 3B, $P<0.05$).

Discussion

The effects of substances regulating spermatogenesis are often determined by morphological analysis of testis tissue. Although this is a powerful approach, it is also time-consuming and requires well-trained observers. Quantifying mRNAs representing specific germ cell types opens the possibility to study the effects of such substances faster. Piwil1 is highly conserved during evolution from insects to mammals as a marker for spermatogonia (Houwing *et al*. 2007), and our immunocytochemical studies showed that Piwil1 protein is particularly highly expressed in the first 3 generations of type A spermatogonia in zebrafish. A similarly restricted expression of *piwil1* mRNA by *in situ* hybridisation has been described by Houwing *et al*. (2009). The axial/lateral element of the synaptonemal complex is encoded by *sycp3l*, and was chosen as a marker for spermatocytes (Yano *et al*. 2008). Moreover, *dazl* was expressed in all spermatogonia and can serve as a marker for all spermatogonia. The DHP-induced changes in expression levels of the germ cells markers were in accordance with our morphological and BrdU-incorporation studies in the *in vivo* trial, suggesting that our molecular approach can serve as a reliable tool to assay stimulatory effects on spermatogenesis.

Another innovative aspect of the present study is the use of an (E_2-induced) androgen insufficiency model to first interrupt spermatogenesis (de Waal *et al*. 2009), before starting the treatment with the compound of interest, DHP in this case, to stimulate spermatogenesis. Since DHP induced an increase in testicular 11*ß*-Hsd activity in eel (Ozaki *et al*. 2006) and zebrafish (Chen *et al*. 2010) *in vitro,* we tested if DHP treatment increased androgen output. Our data did not provide respective evidence but it is possible that a DHP-mediated effect was masked in our experimental system by the E2-induced down-regulation of cyp17a1 expression on both mRNA and protein level (de Waal *et al*. 2009), an enzyme upstream of 11ß-Hsd in the steroidogenic pathway.

Survival and development of germ cells strictly depend on the constant support by somatic cells of the testis, in particular Sertoli cells. Previously, we showed *pgr* mRNA expression in Sertoli cells contacting spermatogonia in zebrafish, and we

hypothesized that DHP might regulate steps in early spermatogenesis indirectly via Sertoli cells. Indeed, three Sertoli cell-expressed genes, *i.e. amh, gsdf* and *igf3*, were up-regulated in testes of animals exposed to DHP *in vivo*. AMH, also known as Müllerian-inhibiting substance, belongs to the TGF-β superfamily; it induces regression of the Müllerian duct in male tetrapod embryos. In addition to this early role during sex differentiation, AMH also influences the development of adult testes by blocking the differentiation of mesenchymal into Leydig cells and by decreasing the expression of steroidogenic enzymes (Josso *et al*. 1998). In teleost fish, which do not form Müllerian ducts (Lasala *et al*. 2004), *amh* genes have been identified in several species (e.g. Japanese eel, Miura *et al*. 2002; Japanese flounder, Yoshinaga *et al*. 2004; zebrafish, Rodríguez-Marí *et al*. 2005; medaka, Klüver *et al*. 2007; rainbow trout, Rolland *et al*. 2009), where they show a male-biased pattern of expression in most species (except medaka), so that Amh may function during testis differentiation. Moreover, androgen-induced (Miura *et al*., 2002) or natural (Rolland *et al*. 2009) initiation of spermatogonial proliferation and differentiation was associated with a down-regulation of *amh* expression, while recombinant Amh suppressed the androgen-induced start of the clonal proliferation of spermatogonia (Miura *et al*. 2002). On the other hand, recombinant eel Amh stimulated the single cell proliferation of medaka germ cells (Shiraishi *et al*. 2008), suggesting that Amh may not only prevent spermatogonial differentiation but may also stimulate expansion of the population of single germ cells. The DHP-induced duplication of testis weight and stimulation of germ cell proliferation and differentiation we observed in zebrafish would be compatible with an Amh-driven expansion of single type A spermatogonia. However, this does not apply to the increased presence of more differentiated germ cell types (type B spermatogonia and spermatocytes) that we have observed as well after DHP exposure. Still, since *amh* mRNA is relatively broadly expressed in zebrafish Sertoli cells (Rodríguez-Marí *et al*. 2005; Schulz *et al*. 2007), *i.e.* also by Sertoli cells contacting late spermatogonial generations, an increased level of *amh* mRNA may in part also reflect the Sertoli cell proliferation that occurs during the successive rounds of spermatogonial mitoses (Schulz *et al*. 2005; Leal *et al*. 2009). The Amh receptor (Amhr) has not been identified in zebrafish but is expressed by Sertoli cells in medaka (Klüver *et al*. 2007). Hence, it is possible that not Amh but Amhr expression determines the proliferation/differentiation response of a given spermatogenic cyst. Finally, different from eel and rainbow trout, where a single wave of largely synchronised spermatogenesis is observed, zebrafish show successive waves of spermatogenesis after puberty, so that Amh and/or Amhr levels may differ from cyst to cyst under normal circumstances, while our experimental model may have induced a synchronized progression through spermatogenesis.

In contrast to *amh*, *gsdf* is a teleost-specific gene. Also Gsdf is a TGF-β superfamily member. Two forms of *gsdf* have been identified recently in rainbow trout and some other salmonid species, while a single form is found in the genome of other fish species (Lareyre *et al*. 2008). Both transcript and protein of Gsdf1 were expressed in somatic cells of the genital ridge surrounding primordial germ cells during embryogenesis, and in both granulosa and Sertoli cells at later stages. In contrast,

gsdf2 transcript is restricted to testis and to the Sertoli cells. Apparently conflicting data exist as regards the possible roles of the Gsdf forms. Sawatari *et al*. (2007) described a stimulatory effect of Gsdf, probably Gsdf2, on spermatogonial proliferation, while Lareyre *et al*. (2008) reported that *gsdf1* mRNA is highly expressed in Sertoli cell surrounding quiescent type A spermatogonia but becomes down-regulated by androgens in association with the start of spermatogenesis. We localised zebrafish *gsdf* mRNA to Sertoli cells surrounding spermatogonia, including large clones of type B spermatogonia, which suggests that Gsdf may have functions in the spermatogonial phase in the zebrafish testis. Up-regulation of *gsdf* mRNA in a DHP-stimulated testis showing high spermatogenic activity and a broad expression pattern of *gsdf* mRNA in most, if not all, Sertoli cells suggests that zebrafisg Gsdf may be more similar to the salmonid Gsdf2, and hence possibly involved in stimulating spermatogonial proliferation.

Insulin-like growth factors (IGFs), including IGF-1 and IGF-2, are evolutionarily conserved peptides across vertebrates and act through a conserved signaling pathway (Wood *et al*. 2005). In fish, due to an additional round of genome duplication, two *igf2* genes (*igf2a* and *igf2b*), and two igf1r genes (igf1ra and igf1rb) have been reported in zebrafish (Sang *et al*. 2008). Moreover, Igf3, which is expressed specifically in gonadal tissue, was discovered in zebrafish, medaka and Nile tilapia, teleost species from different taxonomic orders (Wang *et al*. 2008). Information on the roles of testicular paracrine/autocrin Igfs action in adult spermatogenesis is limited in teleosts. Igf1 is required as permissive factor for androgen-stimulated spermatogenesis in a Japanese eel testis tissue culture system (Nader *et al*. 1999). Igf1 also stimulated the proliferation of rainbow trout spermatogonia in primary cell culture (Loir 1999). Our preliminary data indicated that recombinant sea bream Igf1 stimulated zebrafish spermatogenesis in a primary testis tissue culture system (Leal *et al*. 2006). Information on the regulation of expression and on the bioactivity of other IGF peptides (Igf2a, Igf2b and Igf3) in the testis is scarce. Only one report suggested that Igf3 may be involved in regulating testicular functions in tilapia, as inferred from high levels of testicular expression and their down-regulation by estrogen treatment (Berishvili *et al*. 2010). In the present study, the expression levels of *igf2b*, *igf3* and *igf1rb* were significantly higher in testis showing clearly elevated spermatogenetic activity in response to DHP *in vivo*, which suggests that the Igf signalling pathway is involved in mediating the DHP-stimulated early spermatogenesis, rendering this pathway – next to Gsdf and Amh – interesting candidates for further studies on their roles in zebrafish spermatogenesis.

As a step in this direction, we examined the effect of DHP on these gene expression levels using a recently developed, primary testis tissue culture system for zebrafish (Leal *et al*. 2009). After 7 days *ex vivo* DHP treatment, testicular explants showed significantly increased expression of germ cell marker genes. This is in line with a recent study, in which ex vivo incubation of primary zebrafish testis tissue fragments with DHP resulted in significantly increased incorporation of BrdU (Hanna *et al*. 2010). However, next to the germ cell markers, only *igf1rb* mRNA showed a significant increase, which suggests that DHP acts directly on the testis level to

up-regulate *igf1rb* expression. The observations that (i) up-regulation of germ cell markers was more prominent in the *in vivo* than in the *ex vivo* experiment, and (ii) that except for *igf1rb* mRNA, growth factor and receptor expression did not change in the *ex vivo* experiment may indicate that the DHP-mediated stimulation of spermatogenesis has a relatively weak direct component, and a relatively strong indirect component that becomes obvious in the *in vivo* experiment.

What might be the background for the difference between the *in vivo* and *ex vivo* situation? In a mammalian pituitary cell line, progesterone enhanced FSHβ but repressed LHβ gene expression (Thackray *et al*. 2006). A recent study demonstrated direct effects of androgens and estrogens on pituitary gonadotrophs in zebrafish (Lin and Ge 2009), while there is no information on direct effects of DHP on the brain and/or pituitary level. However, membrane progesterone receptors are expressed in zebrafish brain and pituitary (Hanna and Zhu 2009) and the nuclear Pgr is expressed in the brain (Chen *et al*. 2010) in areas known to be involved in the regulation of reproduction, e.g. in the preoptic region of the hypothalamus (Hanna *et al*. 2010). If also in zebrafish DHP should stimulate *fshb* mRNA expression, the stronger effect of DHP in vivo might reflect a combined effect of DHP and Fsh, the latter being compatible with the elevated expression of Sertoli cell specific genes (e.g. *gsdf* and *amh* mRNA).

In summary, we provide evidence that DHP strongly stimulates spermatogenesis in zebrafish *in vivo*, involving the up-regulation of expression of different growth factors, and we show for the first time that this includes stimulation of early spermatogonial generations. Moreover, *igf1rb* transcripts levels are up-regulated after DHP treatment *in vivo* and *ex vivo*, which suggest that part of the stimulatory effect on spermatogenesis may be mediated via the Igf signalling system.

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References

Berishvili G, Baroiller JF, Eppler E, Reinecke M 2010 Insulin-like growth factor-3 (IGF-3) in male and female gonads of the tilapia: development and regulation of gene expression by growth hormone (GH) and 17*α*-ethinylestradiol (EE2). *General and Comparative Endocrinology* **167** 128-134.

Bontems F, Stein A, Marlow F, Lyautey J, Gupta T, Mullins MC, Dosch R 2009 Bucky ball organizes germ plasm assembly in zebrafish. *Current Biology* **19** 414-422.

Chen SX, Bogerd J, García-López A, de Jonge H, de Waal PP, Hong WS, Schulz RW 2010 Molecular cloning and functional characterization of a zebrafish nuclear progesterone receptor. *Biology of Reproduction* **82** 171-181.

Conneely OM, Mulac-Jericevic B, DeMayo F, Lydon JP, O'Malley BW 2002 Reproductive functions of progesterone receptors. *Recent Progress in Hormone Research* **57** 339-355.

de Waal PP, Leal MC, García-López A, Liarte S, de Jonge H, Hinfray N, Brion F, Schulz RW, Bogerd J 2009 Oestrogen-induced androgen insufficiency results in a reduction of proliferation and differentiation of spermatogonia in the zebrafish testis. *Journal of Endocrinology* **202** 287-297.

Dickey JT, Swanson P 1998 Effects of sex steroids on gonadotropin (FSH and LH) regulation in coho salmon (*Oncorhynchus kisutch*). *Journal of Molecular Endocrinology* **21** 291-306.

García-López A, de Jonge H, Nóbrega RH, de Waal PP, van Dijk W, Hemrika W, Taranger GL, Bogerd J, Schulz RW 2010 Studies in zebrafish reveal unusual cellular expression patterns of gonadotropin receptor messenger ribonucleic acids in the testis and unexpected functional differentiation of the gonadotropins. *Endocrinology* **151** 2349-2360.

Hanna RN, Zhu Y 2009 Expression of membrane progestin receptors in zebrafish (*Danio rerio*) oocytes, testis and pituitary. *General and Comparative Endocrinology* **161** 153-157.

Hanna RN, Daly SC, Pang Y, Anglade I, Kah O, Thomas P, Zhu Y 2010 Characterization and expression of the nuclear progestin receptor in zebrafish gonads and brain. *Biology of Reproduction* **82** 112-122.

Houwing S, Kamminga LM, Berezikov E, Cronembold D, Girard A, van den Elst H, Filippov DV, Blaser H, Raz E, Moens CB, Plasterk RHA, Hannon GJ, Draper BW, Ketting RF 2007 A role for piwi and piRNAs in germ cell maintenance and transposon silencing in zebrafish. *Cell* **129** 69-82.

Ikeuchi T, Todo T, Kobayashi T, Nagahama Y 2002 A novel progesterone receptor subtype in the Japanese eel, *Anguilla japonica*. *FEBS Letters* **510** 77-82.

Josso N, Racine C, di Clemente N, Rey R, Xavier F 1998 The role of anti-Müllerian hormone in gonadal development. *Molecular and Cellular Endocrinology* **145** 3-7.

Klüver N, Pfenning F, Pala I, Storch K, Schlider M, Froschauer A, Gutzeit HO, Schartl M 2007 Differential expression of anti-Müllerian hormone (amh) and anti-Müllerian hormone receptor type II (amhrII) in the teleost medaka. *Developmental Dynamics* **236** 271-281.

Lareyre JJ, Ricordel MJ, Mahé S, Goupil AS, Vizziano D, Bobe J, Guiguen Y, LeGac F 2008 Two new TGF beta members are restricted to the gonad and differentially expressed during sex differentiation and gametogenesis in trout. *Cybium* **Suppl. 32** 133-135.

Lasala C, Carre-Eusebe D, Picard JY, Rey R 2004 Subcellular and molecular mechanisms regulating anti-Müllerian hormone gene expression in mammalian and nonmammalian species. *DNA and Cell Biology* **23** 572-585.

Leal MC, Skaar KS, de Françac LR, Schulz RW 2006 The effects of IGF-I on zebrafish testis in tissue culture. *Animal Reproduction: Proceedings of the International Symopsium on Animal biology of Reproduction, Nov, 15-18, 2006,*

Belo Horizonte, MG, Brazil **3** 181.

Leal MC, de Waal PP, García-López A, Chen SX, Bogerd J, Schulz RW 2009 Zebrafish (*Danio rerio*) primary testis tissue culture: an approach to study testis function *ex vivo*. *General and Comparative Endocrinology* **162** 134-138.

Lin SW, Ge W 2009 Differential regulation of gonadotropins (FSH and LH) and growth hormone (GH) by neuroendocrine, endocrine, and paracrine factors in the zebrafish--an *in vitro* approach. *General and Comparative Endocrinology* **160** 183-193.

Loir M 1999 Spermatogonia of rainbow trout: II. in vitro study of the influence of pituitary hormones, growth factors and steroids on mitotic activity. Molecular *Reproduction and Devlopment* **53** 434-442.

Miura T, Yamauchi K, Takahashi H, Nagahama Y 1992 The role of hormones in the acquisition of sperm motility in salmonid fish. *The Journal of Experimental Zoology* **261** 359-363.

Miura T, Miura C, Konda Y, Yamauchi K 2002 Spermatogenesis-preventing substance in Japanese eel. *Development* **129** 2689-2697.

Miura T, Higuchi M, Ozaki Y, Ohta T, Miura C 2006 Progestin is an essential factor for the initiation of the meiosis in spermatogenetic cells of the eel. *Proceedings of the National Academy of Sciences of the United States of America* **103** 7333-7338.

Miura C, Ohta T, Ozaki Y, Tanaka H, Mirua T 2009 Trypsin is a multifunctional factor in spermatogenesis. *Proceedings of the National Academy of Sciences of the United States of America* **106** 20972-20977.

Nader MR, Miura T, Ando N, Miura C, Yamauchi K 1999 Recombinant human insulin-like growth factor I stimulates all stages of 11-ketotestosterone-induced spermatogenesis in Japanese eel, Anguilla japonica, in vitro. *Biology of Reproduction* **61** 944-947.

Ozaki Y, Higuchi M, Miura C, Yamaguchi S, Tozawa Y, Miura T 2006 Roles of 11*β*-hydroxysteroid deheydrogenase in fish spermatogenesis. *Endocrinology* **147** 5139-5146.

Rodríguez-Marí A, Yan Y-L, BreMiller RA, Wilson C, Canestro C, Postlethwait JH 2005 Characterization and expression pattern of zebrafish *anti-Müllerian hormone* (*amh*) relative to *sox9a*, *sox9b*, and *cyp19a1a*, during gonad development. *Gene Expression Patterns* **5** 655–667.

Rolland AD, Lareyre JJ, Goupil AS, Montfort J, Ricordel MJ, Esquerré D, Hugot K, Houlgatte R, Chalmel F, Le Gac F 2009 Expression profiling of rainbow trout testis development identifies evolutionary conserved genes involved in spermatogenesis. *BMC Genomics* **10** 546.

Sang X, Curran MS, Wood AW 2008 Paracrine insulin-like growth factor signaling influences primordial germ cell migration: in vivo evidence from the zebrafish model. *Endocrinology* **149** 5035-5042.

Sawatari E, Shikina S, Takeuchi T, Yoshizaki G 2007 A novel transforming growth factor-beta superfamily member expressed in gonadal somatic cells enhances primordial germ cell and spermatogonial proliferation in rainbow trout

(*Oncorhynchus mykiss*). *Developmental Biology* **301** 266-275.

Schmittgen TD, Livak KJ 2008 Analyzing real-time PCR data by the comparative C_T method. *Nature Protocols* **3** 1101-1108.

Schulz RW, Bogerd J, Male R, Ball J, Fenske M, Olsen LC, Tyler CR 2007 Estrogen-induced alterations in amh and dmrt1 expression signal for disruption in male sexual development in the zebrafish. *Environmental Science and Technology* **41** 6305-6310.

Schulz RW, Menting S, Bogerd J, de Françac LR, Vilela DAR, Godinho HP 2005 Sertoli cell proliferation in the adult testis: evidence from two fish species belonging to different orders. *Biology of Reproduction* **73** 891-898.

Schulz RW, van der Corput L, Janssen-Dommerholt C, Goos HJTh 1994 Sexual steroids during puberty in male African catfish (*Clarias gariepinus*): serum levels and gonadotropin-stimulated testicular secretion *in vitro*. *Journal of comparative physiology. B, Biochemical, systemic, and environmental physiology* **164** 195-205.

Shiraishi E, Yoshinaga N, Miura T, Yokoi H, Wakamatsu Y, Abe S, Kitano T 2008 Mullerian inhibiting substance is required for germ cell proliferation during early gonadal differentiation in medaka (*Oryzias latipes*). *Endocrinology* **149** 1813-1819.

Thackray VG, McGillivray SM, Mellon PL 2006 Androgens, progestins and glucocorticoids induce follicle-stimulating hormone -subunit gene expression at the level of the gonadotrope. *Molecular Endocrinology* **20** 2062-2079.

Thomas P 2008 Characteristics of membrane progestin receptor alpha (mPRα) and progesterone membrane receptor component 1 (PGMRC1) and their roles in mediating rapid progestin actions. *Frontiers in Neuroendocrinology* **29** 292-312.

Thomas P, Tubbs C, Detweiler C, Das S, Ford L, Breckenridge-Miller D 2005 Binding characteristics, hormonal regulation and identity of the sperm membrane progestin receptor in Atlantic croaker. *Steroids* **70** 427-433.

Ueda H, Kanbegawa A, Nagahama Y 1985 Involvement of gonadotrophin and steroid hormones in spermiation in the amago salmon, *Oncorhynchus rhodurus*, and goldfish, *Carassius auratus*. *General and Comparative Endocrinology* **59** 24-30.

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F 2002 Accurate normalization of real-time quantitative QPCR data by geometric averaging of multiple internal controls. *Genome Biology* **3** research0034.1-0034.12.

Vischer HF, Teves ACC, Ackermans JCM, van Dijk W, Schulz RW, Bogerd J 2003 Cloning and spatiotemporal expression of the follicle-stimulating hormone β subunit complementary cDNA in the African catfish (*Clarias gariepinus*). *Biology of Reproduction* **68** 1324-1332.

Wang D, Jiao B, Hu C, Huang X, Liu Z, Cheng CH 2008 Discovery of a gonadspecific IGF subtype in teleost. *Biochemical and Biophysical Research Communications* **367** 336-341.

Westerfield M 2000 The zebrafish book: a guide for the laboratory use of zebrafish

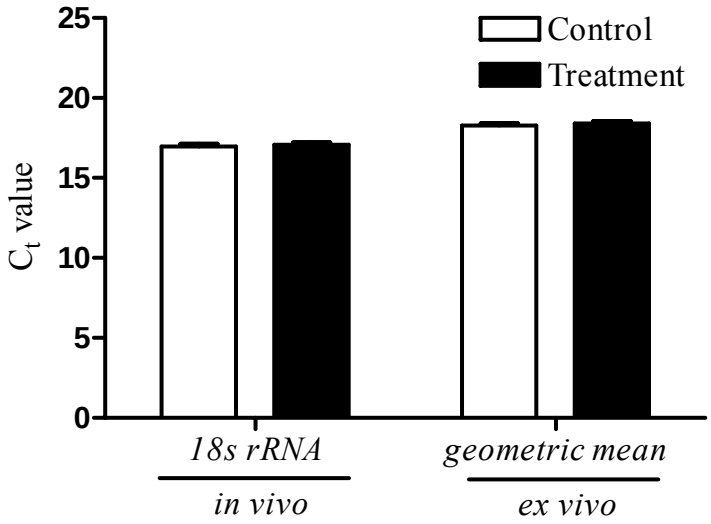
(*Danio rerio*). 4th edn. Eugene: University of Oregon Press.

Wood AW, Duan C, Bern HA 2005 Insulin-like growth factor signaling in fish. *International Review of Cytology* **243** 215-285.

Yano A, Suzuki K, Yoshizaki G 2008 Flow-cytometric isolation of testicular germ cells from rainbow trout (*Oncorhynchus mykiss*) carrying the green fluorescent protein gene driven by trout *vasa* regulatory regions. *Biology of Reproduction* **78** 151-158.

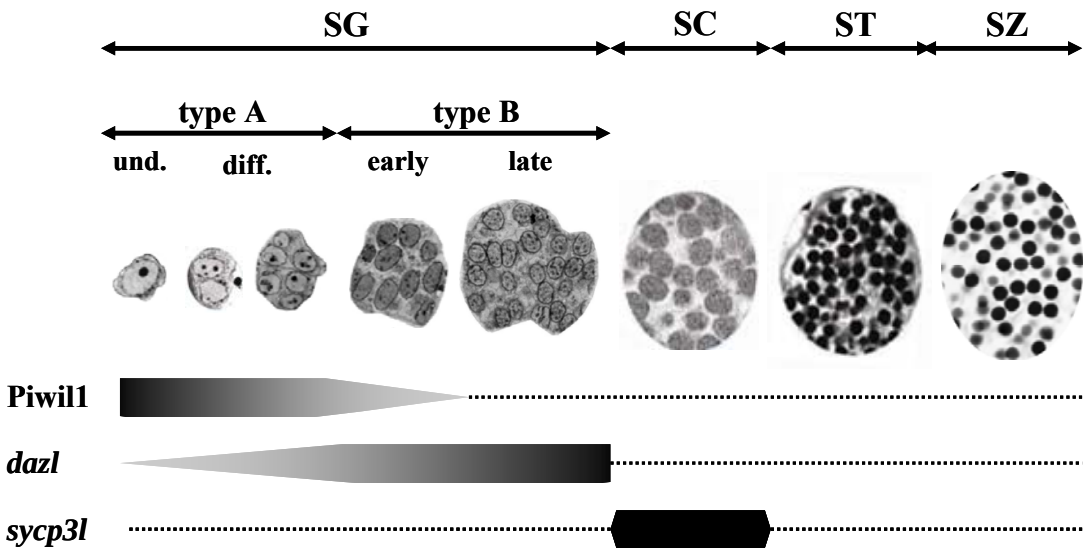
Yoshinaga N, Shiraishi E, Yamamoto T, Iguchi T, Abe S, Kitano T 2004 Sexually dimorphic expression of a teleost homologue of Müllerian inhibiting substance during gonadal sex differentiation in Japanese flounder, *Paralichthys olivaceus*. *Biochemical and Biophysical Research Communications* **322** 508-513.

Supplemental information

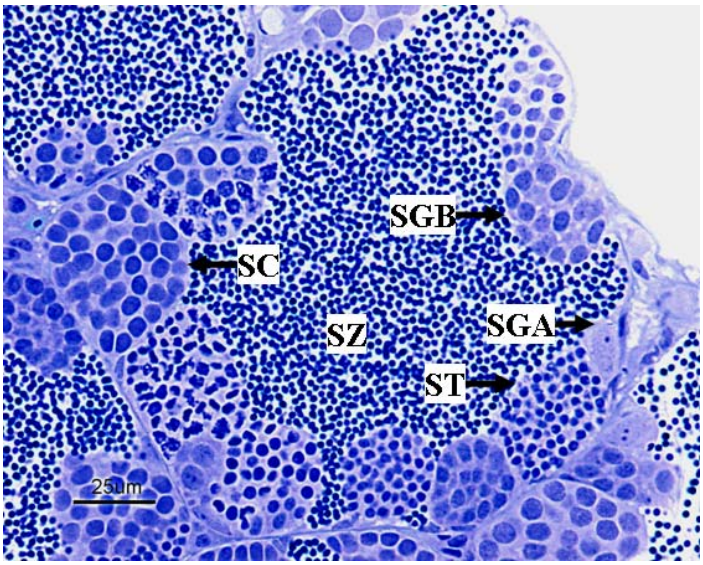


Supplemental figure 1 *18s rRNA* and geometric mean of three internal control genes (*18s rRNA, ef1α* and *bactin1*) from *in vivo* and *ex vivo* experiments.

No significant differences (P>0.05) in testicular *18s rRNA* and geometric mean from *in vivo* and *ex vivo* experiments, respectively.



Supplemental figure 2 Expression of Piwil1 protein, and *dazl* and *sycp3l* transcripts during zebrafish spermatogenesis. As described in Houwing *et al*. (2009), *piwil1* mRNA shows a similar expression restricted to early spermatogonia.
SG, spermatogonia; SC, spermatocytes; ST, spermatid; SZ, spermatozoa; und., undifferentiated type A spermatogonia; diff., differentiated type A spermatogonia.



Supplemental figure 3 Cross section of zebrafish testis.
A plastic section of glutaraldehyde fixed, freshly excised zebrafish testis tissue showing all germ cell stages. SGA, type A spermatogonia; SGB, type B spermatogonia; SC, spermatocytes; ST, spermatid; SZ, spermatozoa.

Supplemental table 1 Primers and TaqMan fluorogenic probes used to quantify mRNA levels by qPCR analysis in zebrafish testicular samples.

Target	GenBank accession no.	Primer*	Sequence (5'→3')*
piwil1	NM_183338	2542 (Fw)	GATACCGCTGCTGGAAAAAGG
		2543 (Rv)	GCAAGACACACTTGGAGAACCA
dazl	AB018191	3104 (Fw)	AGTGCAGACTTTGCTAACCCTTATGTA
		3105 (Rv)	GTCCACTGCTCCAAGTTGCTCT
sycp3l	BC115343	2730 (Fw)	AGAAGCTGACCCAAGATCATTCC
		2731 (Rv)	AGCTTCAGTTGCTGGCGAAA
star	NM_131663	2546 (Fw)	CCTGGAATGCCTGAGCAGAA
		2547 (Rv)	ATCTGCACTTGGTCGCATGAC
gsdf	BC045846	2366 (Fw)	CATCTGCGGGAGTCATTGAAA
		2367 (Rv)	CAGAGTCCTCCGGCAAGCT
igf1	BX510924	2394 (Fw)	CCCAGGACACCAAAGAAACCTA
		2395 (Rv)	CGGCTCGAGTTCTTCTGATGA
igf2b	AL954320	3077 (Fw)	CTGCCATGGATGATTACCATGTATT
		3078 (Rv)	CATGGACAATGACAGAACGAAGAC
igf3	CT025854	2680 (Fw)	TGTGCGGAGACAGAGGCTTT
		2681 (Rv)	CGCCGCACTTTCTTGGATT
iIgf1ra	BX470160	2362 (Fw)	TACATCGCTGGCAACAAGCA
		2363 (Rv)	TCATTGAAACTGGTCCTTATGCAAT
igf1rb	AL928976	2595 (Fw)	GTGCTGGTCCTCTCCACACTCT
		2596 (Rv)	TTACCGATGTCGTTGCCAATATC
bactin1	AL928650	2647 (Fw)	TGCTCTGTATGGCGCATTGA
		2648 (Rv)	GCTCCTCCCCCTGTTAGACAAC
ef1α	L47669	AG (Fw)	GCCGTCCCACCGACAAG
		AH (Rv)	CCACACGACCCACAGGTACAG
		AI (probe)	CTCCAATTTTGTACACATCCTGAAGTGGCA
amh	AY721604	AD (Fw)	CTCTGACCTTGATGAGCCTCATTT
		AE (Rv)	GGATGTCCCTTAAGAACTTTTGCA
		AF (probe)	ATTCCACAGGATGAGAGGCTCCCATCC

*Sequences are shown for the sense (Fw) and antisense (Rv) primers, and the TaqMan probe (probe). Probes have a 6-FAM label at their 5'-ends, and a TAMRA quencher label at their 3'-ends.

Chapter 4

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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 progesterone during early stages of spermatogenesis in fish, we carried out studies on the nuclear progesterone receptor (Pgr) of the Atlantic salmon. The open-reading frame of this *pgr* consists of 2157 bp, coding for a 718 amino acids-long protein that shows the highest similarity with other piscine Pgr proteins. Functional characterization of the receptor expressed in mammalian cells revealed that salmon Pgr exhibited progesterone-specific, dose-dependent induction of reporter gene expression, with 17*α*,20*β*-dihydroxy-4-pregnen-3-one (DHP), a typical piscine progesterone, showing the highest potency. Expression of *pgr* mRNA was significantly higher in testis than in ovary. In testis at the beginning of the pubertal growth phase, *pgr* mRNA was restricted to Sertoli cells surrounding spermatogonia. We then analyzed testicular *pgr* mRNA and DHP plasma levels in samples collected from animals during the onset of spermatogenesis that were exposed to natural light or to constant light. Photoperiod manipulation resulted, as expected, in significant differences in testis growth, but grouping of the animals according to their progress in spermatogenesis (i.e. type A spermatogonia, type B spermatogonia, or primary spermatocytes as most advanced germ cell type) showed that both plasma DHP levels and *pgr* mRNA levels increased in fish where germ cell development had reached the stage of late type B spermatogonia, and further increased when germ cells entered meiosis, i.e. when spermatocytes were present. Our data suggests a role of Pgr in mediating DHP-stimulated, relatively early steps in spermatogenesis in Atlantic salmon, such as the differentiation of late type B spermatogonia and the entry into meiosis.

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 was reported during the progression of spermatogonial proliferation (Dépêche & Sire 1982, Scott & Sumpter 1989, Vizziano *et al*. 1996), suggesting that DHP plays a role also during early stages of spermatogenesis than during spermiation and spawning. Indeed, DHP induced the initiation of meiosis in male germ cells in Japanese eel (*Anguilla japonica*) (Miura *et al*. 2006).

The biological activity of progesterone is mediated via specific receptors. Nuclear and membrane-bound progestin 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*), which is expressed in germ cells, mediates the non-genomic actions of progestins to induce sperm hypermotility in a number of teleost species (Tubbs & Thomas 2008). In Japanese eel, *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, data from another group 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 another, unrelated order, the Salmoniformes, we isolated a full-length *pgr* cDNA from Atlantic salmon (*Salmo salar* L.). After pharmacological characterization of the salmon Pgr, we examined the *pgr* mRNA expression pattern in different adult salmon tissues. To investigate the possible role of Pgr in early stages of spermatogenesis, we then 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.

Materials and methods

Maintenance, photoperiod treatment, and sampling of fish

Previously immature, two seawinter-old salmon had been kept in sea cages at the Institute of Marine Research (Matre, Norway; 61°N) under natural light (NL) conditions for 19 months until the start of the trial. An initial control sample was collected on January 8. Starting on February 1, half of the animals were exposed to additional constant light (LL), while the other half remained under NL conditions. Samples were then collected at 4 time points: February 18, March 19, April 25, and June 11. 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 anaesthetized with 6 ppt metomidate (Syndel, Victoria, BC, Canada), weighed (total body weight), blood was collected in heparinized syringes from the caudal veins and gonads were excised and weighed. The gonado-somatic index (GSI) was calculated as: GSI (%) = gonad weight (g) x100 / total body weight (g). Testis tissue samples were either shock frozen in liquid nitrogen and then stored at -80ºC 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 three stages of spermatogenesis. In stage SG-A, the testes contained only type A spermatogonia that show a large nucleus (~10 μm; see supplemental Fig. 1A) with little heterochromatin and one or two prominent nucleoli. This largest type of spermatogonia often occurs as single cells and are then in contact with one or two Sertoli cells. In stage SG-B, type B spermatogonia are present in addition to type A spermatogonia. Early generations of type B spermatogonia occur in pairs or small groups and have a smaller nucleus (~7 μm; see supplemental Fig. 1B) that is stained more intensely with hematoxylin. Late type B spermatogonia occur in larger groups and show an increased amount of heterochromatin speckles distributed over an in general more darkly stained nucleus (see supplemental Fig. 1C), and the cytoplasmic area is staining more intensely with eosin than in the previous germ cell generations. In stage SC, spermatocytes are present, which occur in large groups and are characterized by the presence of darkly staining meiotic chromosomes at different stages of condensation during the first meiotic prophase (see supplemental Fig. 1D). In two out of the 12 animals found in stage SC, spermatids were found as well (see supplemental Fig. 1D).

Aliquots of blood plasma were obtained by centrifugation at 5000 rpm 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 was extracted with 5 ml diethyl ether and shaken vigorously for 20 sec, and the ether was decanted. This procedure was repeated twice, the ether fractions were combined and evaporated to dryness. The residue was dissolved in 5 ml 70% (v/v) methanol, and stored at -20°C over night for defatting. After centrifugation at 5000 rpm 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 dT_{25} (Dynal A.S., Oslo, Norway) and reverse transcribed to 5'- and 3'-RACE ready cDNA using a SMART RACE cDNA amplification kit (Clontech, Mountain View, CA, USA) following the manufacturers' instructions.

To obtain a partial salmon *pgr* cDNA sequence, 2 µl random hexamer-primed salmon ovary cDNA was used as template in a PCR with primer set 2699 and 2700 (see Supplemental Table 1), 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, Breda, The Netherlands) and plasmid DNA of 2 clones was prepared for DNA sequence analysis.

To isolate the 5'- and 3'-ends of the salmon *pgr* cDNA, gene-specific primers (primer 2722 and 2720; Supplemental Table 1) based on the consensus nucleotide sequence of the 2 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 (primers 2721 and 2723; see Supplemental 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 (Supplemental 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 open-reading frame of the salmon *pgr* was PCR amplified using primer 2794 and 2796 (Supplemental 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 percentages identity were calculated. We only selected (deduced) PGR amino acid sequences from studies that experimentally demonstrated progesterone binding to the receptors (see Supplemental 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 (unpublished data).

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 Dulbecco modified Eagle medium (DMEM) supplemented with 10% v/v fetal bovine serum (FBS), glutamine, and penicillin/streptomycin (Gibco, Breda, The Netherlands) at 37°C in a 5% CO_2 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, U.S.A.). Each compound was tested in three independent experiments using cells from different transfections.

The following steroids were used in this study: DHP, 20*β*-S, progesterone (P4), 17*α*(OH)P4, testosterone (T), 11-ketotestosterone (11-KT), 17*β*-estradiol (E_2), 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 cryo-sections prepared from paraformaldehyde-fixed testis tissue from fish sampled during the onset of spermatogenesis. Cell nuclei were visualized with a DAPI counterstaining (Vectashield with DAPI, Vector Laboratories). Specific primers (3168 and 3198; Supplemental Table 1) were designed to PCR 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 (Supplemental Table 1) were designed to detect salmon *pgr* using quantitative real-time PCR (qPCR). The specificity and efficiency of this *pgr* primer set was tested with qPCR on serial dilutions of salmon testis cDNA as described elsewhere (Vischer *et al*. 2003). Atlantic salmon elongation factor 1*α* (*ef1α*) was used as reference gene as described in detail previously (Andersson *et al*. 2009); no significant differences in expression levels were found in the testes samples analyzed (Supplemental Figure 5). All real-time, quantitative PCRs (qPCR) were performed in 20 µl reactions and C_t values 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 *ef1α* mRNA levels, and shown relative to the tissue showing the highest level of expression (immature testis tissue), which was set to 1. Expression levels in testis tissue were normalized to *ef1a* mRNA, corrected according to Kusakabe *et al*. (2006) for RNA yield (μg RNA recovered per mg tissue extracted), testis mass and body weight, and 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 (see above: stages SG-A, SG-B, and SC) and exposure to NL or LL. Data were subjected to analysis of variance, followed by a Tukey unequal N HSD test to identify differences among groups. Differences between testes in the SG-A and SG-B stage, 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 ± SEM.

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 (Supplemental Fig. 2). Comparison of the deduced amino acid sequence of the salmon *pgr* with PGRs from other species is shown in Supplemental Table 2. The salmon Pgr amino acid sequence could be subdivided into 4 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 (Supplemental Fig. 3). 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 EC_{50} value being DHP (17.9±0.2 nM). Also at a fixed concentration of 1 μM, DHP and 20*β*-S were the most potent inducers of luciferase activity (4-fold and 2 fold above control, respectively; Fig. 1B). The other

three progesterone-related hormones tested elicited increases of luciferase activity only at a concentration of 10 μM, while other steroid hormones assayed (T, 11-KT, E_2, or cortisol) were ineffective at 10 μM.

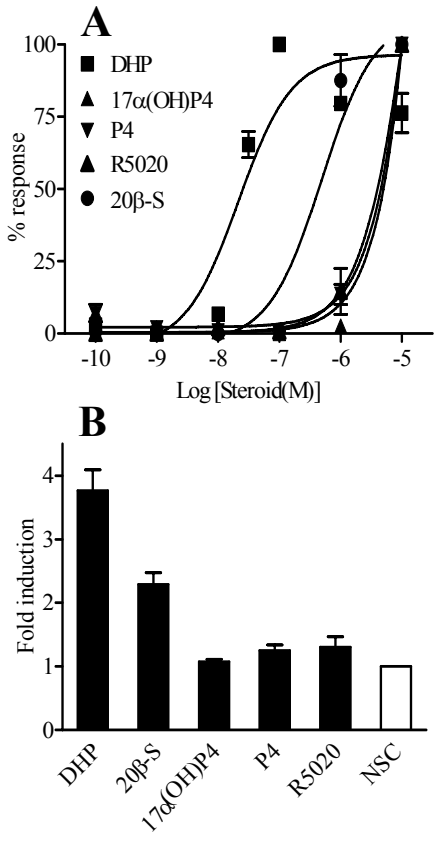

Figure 1 Ligand-induced transactivation properties of the Atlantic salmon Pgr. HEK 293T cells were transiently co-transfected with the pGL3-MMTV-Luc and the salmon *pgr* expression vector constructs. (A) Transfected cells were incubated with increasing concentrations (from 0.1 nM to 10 μM) of various progesterones. Percentage (%) of response: values are given relative to the maximal amount of luciferase activity for each condition. Each point represents the mean ± SEM 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 μM 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 SEM.

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).

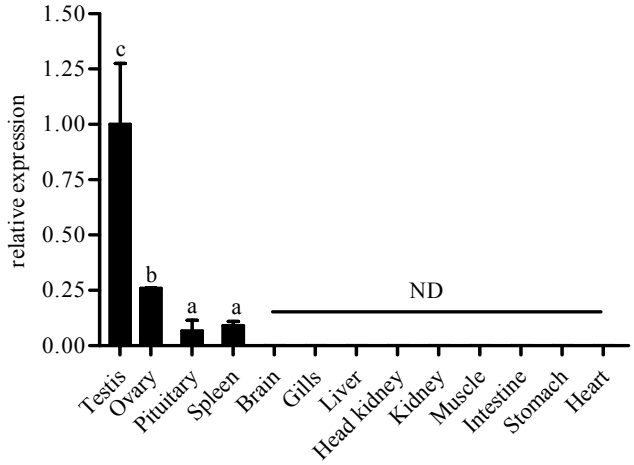

Figure 2 Relative expression of *pgr* mRNA in immature testis and in different tissues of immature female Atlantic salmon. The *pgr* mRNA expression levels were normalized to the expression of *ef1α* mRNA. Values represent mean ± SEM (n=3) relative to testicular *pgr* mRNA levels. Bars marked with different letters are significantly different from each other (P<0.05). ND, not detectable.

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 Sertoli cells surrounding spermatogonia (Fig. 3A, 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, D).

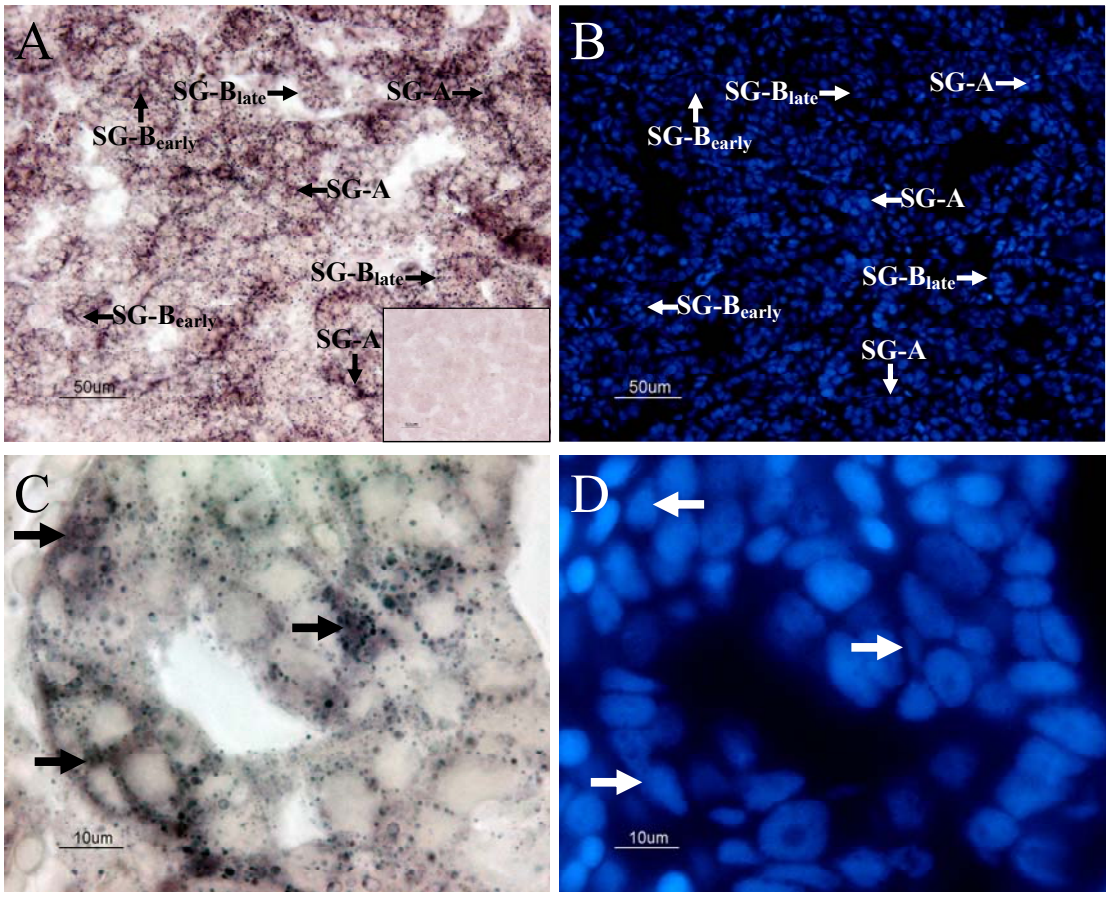

Figure 3 *In situ* hybridization of salmon *pgr* mRNA in testis from an NL-exposed male at the beginning of spermatogenesis, containing type A, early type B, and late type B spermatogonia. The antisense cRNA probe (A) strongly labeled Sertoli cells contacting type A (SG-A) and early type B spermatogonia (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 did not result in specific staining.

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

All males kept under NL conditions were progressively recruited into maturation until June. In ~40% of the LL-exposed males, on the other hand, testis growth was blocked (supplemental Figure 4) while in those individuals maturing under LL-conditions, testis growth exceeded the growth found under NL-conditions (Fig. 4A, B).

When NL-exposed animals were grouped according to the stage of germ cell development, the GSI and DHP plasma levels were low when only SG-A were present in the testis, but increased with progressing spermatogenesis (Fig. 4A, C). Testicular *pgr* mRNA levels also showed this pattern (Fig. 4E). Analyzing stage SG-B more closely, we detected two subgroups. In males sampled in February, only early type B spermatogonia were observed, while all males sampled in March (or later) showed early and late type B spermatogonia. Considering GSI, plasma DHP as well as testicular *pgr* mRNA levels, we found that males with early type B spermatogonia were similar to males in the previous stage of development (i.e. stage SG-A). We therefore conclude that the increases in stage SG-B observed in all parameters analyzed, is associated with the appearance of late type B spermatogonia.

Grouping of the LL-exposed animals according to the stage of spermatogenesis resulted in plasma DHP (Fig. 4D) and testicular *pgr* expression pattern (Fig. 4F) that were very similar to those observed under NL conditions. It seems, therefore, that for the changes in hormone level and receptor expression the stage development is more relevant than the time of sampling. This is particularly evident in stage SG-A, composed of males found at all sampling dates.

Nuclear *pgr* mRNA expression in testicular samples collected from animals under NL or LL conditions was evaluated according to the $\Delta\Delta C_t$ method, using *ef1α* mRNA levels for normalization. It is therefore important to note that the *ef1α* mRNA levels were stable throughout the experimental period and showed no significant differences between sampling points or treatment groups (Supplemental Figure 5).

Discussion

The structural features of the cloned salmon *pgr* cDNA suggest 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 D box (AGRND) sequences (Umesono & Evans 1989), important regions for the recognition of Pgr target gene sequences, which 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 (Chen *et al*. 2010, Todo *et al*. 2000, Ikeuchi *et al*. 2002), so that this particular region may not be available for Pgr-mediated Mos/MAPK activation in teleosts.

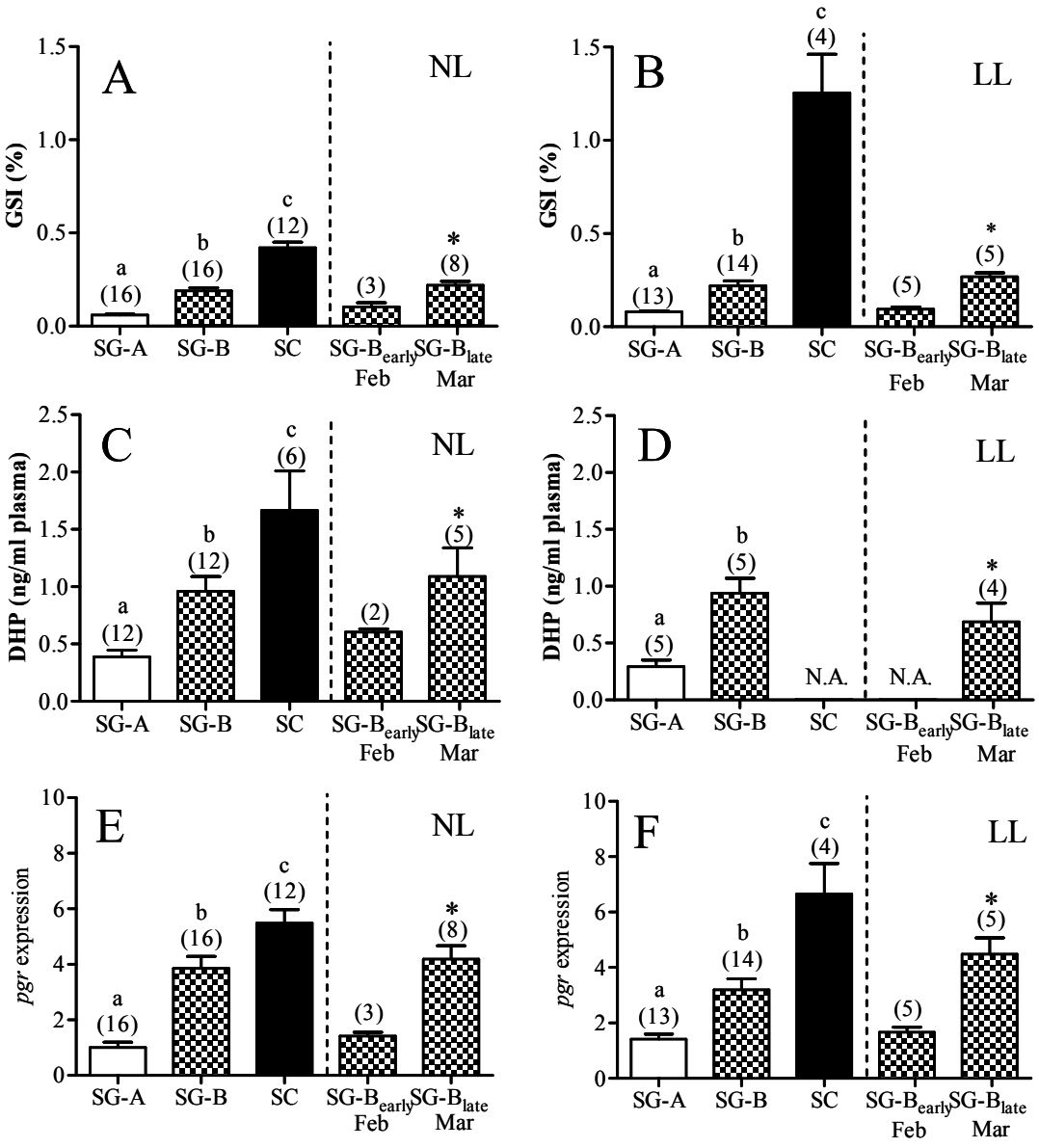

Figure 4 Changes in GSI (A, B), plasma DHP (C, D) and testicular *pgr* mRNA expression (E, F) levels in Atlantic salmon exposed to natural light (NL) (A, C, E) or to additional constant light (LL) (B, D, F) during the onset of spermatogenesis. To the left of the dashed line, males were assigned to groups 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 developed germ cell type in the testis, and of males sampled in March showing in addition late type B spermatogonia (SG-B_{late}). Asterisk indicates significant difference to testes showing SG-A and to testes showing only 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.

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, Takefugu, Tetraodon, and stickleback (Chen *et al*. 2009, 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, P4 is considered to be the main ligand for their PGRs. In teleost fish, however, DHP is the main ligand for Pgrs (Ikeuchi *et al*. 2002, Chen *et al*. 2010). In the present study, we demonstrated that the salmon *pgr* cDNA codes for a functional Pgr, which is able to transactivate target genes in a progesterone-dependent manner. Transactivation was progesterone-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 reproductive organ (Graham & Clarke 1997, González-Morán *et al*. 2008, Wang *et al*. 2004). 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 have 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 and progesterone receptor 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 (Kohler *et al*. 2007; Galena *et al*. 1974; Sirivaidyapong *et al*. 2001); 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 the present study, we found a strong *in situ* hybridization signal of salmon *pgr* mRNA in Sertoli cells surrounding type A and early type B spermatogonia. In Japanese eel, the molecular mechanism underlying DHP-mediated stimulation of spermatogenesis involved elevated expression of 11*β*-hydroxysteroid dehydrogenase (short form) and trypsinogen in Leydig and Sertoli cells, respectively (Ozaki *et al*. 2006, Miura *et al*. 2009). While the Sertoli cell expression we report is consistent with the presence of Pgr in testicular somatic cell in vertebrates, 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).

A major aim of this study was to examine, if *pgr* mRNA amounts and plasma DHP levels vary with changes in (natural or photoperiod-induced) testis growth and spermatogenic activity during the onset of puberty in Atlantic salmon. In seasonally breeding teleosts, the size and cellular composition of the testis changes considerably (Schulz *et al.* 2010) accompanied by a significant change in the amount of total RNA per testes (Kusakabe *et al*. 2002). Therefore, a correction method to report qPCR data for relative gene expression should be developed from a physiological point of view. The proportion of somatic cell transcripts in the total testis mRNA becomes under-represented during the process of spermatogenesis, an effect that fades out again when most germ cells have developed into spermatozoa that contain little RNA. Kusakabe et al. (2006) described an approach that used the RNA yield (μg RNA recovered per mg tissue extracted), total organ mass, and body weight, to account for these changes in gene expression related to tissue growth and cellular representation. This approach estimated the relative level of target gene expression by first calculating the relative amount of target gene expression per testis, and then correct for changes in testicular somatic cell number by normalization to the body weight. Information on the relation between body weight and testicular somatic cell number is not available in salmon, but studies in Nile tilapia and zebrafish showed that Sertoli cell proliferation occurs primarily during spermatogonial proliferation, leveled off during meiosis, and had stopped when Sertoli cells were in contact with spermatids

(Schulz *et al*. 2005, Leal *et al*. 2009). Since testis samples were collected from animals at an early spermatogenic stage in the present study, when testicular somatic cell proliferation accompanies testicular and somatic growth, we presented the *pgr* mRNA expression data after calculations according to Kusakabe *et al*. (2006).

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 the present study, where we found a significant increase of plasma DHP levels from stage SG-B to stage SC in Atlantic salmon. Also, the *pgr* mRNA level increased at that time, suggesting that DHP-activated Pgr may be functionally related to the initiation of meiosis, as had been demonstrated experimentally for Japanese eel (Miura *et al*. 2006).

Another function of DHP during early spermatogenesis was first suggested by Dépêche & Sire (1982), who reported that rainbow trout testis tissue produced DHP from 17*α*(OH)P4 when they were about to start with rapid spermatogonial proliferation at the beginning of testis growth. 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 the present study, analyzing samples collected from animals during the early spermatogenic period, we observed that both *pgr* mRNA and DHP plasma levels were significantly increased in testes in the SG-B stage in comparison with testes in the SG-A stage. 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 and testicular *pgr* mRNA levels. 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. Accordingly, the absence of a coordinated up-regulation of DHP plasma and testicular *pgr* mRNA levels in males that did not yet start (in the NL group), or were blocked (in the LL group), to initiate testis growth, may be a characteristic component of their status of immaturity. Since our in situ hybridization experiments 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. Finally, the strong signals obtained for Sertoli cells in contact with type A and early type B spermatogonia suggests that an increased testicular *pgr* expression level reflects an increased number of early spermatogenic cysts, and also that once DHP participated in stimulating the transition to late type B spermatogonia, *pgr* mRNA expression may become down-regulated in Sertoli cells now associated with late type B spermatogonia.

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 spermatogoina. Further studies using models that allow more direct experimental approaches are needed to develop further studies on Pgr involvement in DHP-stimulated early spermatogenesis.

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References

Amer MA, Miura T, Miura C, Yamauchi K 2001 Involvement of sex steroid hormones in the early stages of spermatogenesis in Japanese huchen (*Hucho perryi*). *Biology of Reproduction* **65** 1057-1066.

Andersson E, Nijenhuis W, Male R, Swanson P, Bogerd J, Taranger GL, Schulz RW 2009 Pharmacological characterization, localization and quantification of expression of gonadotropin receptors in Atlantic salmon (*Salmo salar* L.) ovaries. *General and Comparative Endocrinology* **163** 329-339.

Baynes SM, Scott AP 1985 Seasonal variations in parameters of milt production and in plasma concentration of sex steroids of male rainbow trout (*Salmo gairdneri*). *General and Comparative Endocrinology* **57** 150-160.

Bogerd J, Blomenrohr M, Andersson E, van der Putten HHAGM, Tensen CP, Vischer HF, Granneman JC, Janssen-Dommerholt C, Goos HJTh, Schulz RW 2001 Discrepancy between molecular structure and ligand selectivity of a testicular follicle-stimulating hormone receptor of the African catfish (*Clarias gariepinus*). *Biology of Reproduction* **64** 1633-1643.

Boonyaratanakornkit V, Scott MP, Ribon V, Sherman L, Anderson SM, Maller JL, Miller WT, Edwards DP 2001 Progesterone receptor contains a proline-rich motif that directly interacts with SH3 domains and activates c-Src family of tyrosine kinases. *Molecular Cell* **8** 269-280.

Carroll RL 1988 Vertebrate paleontology and evolution. New York: WH Freeman and Company.

Chen SX, Bogerd J, García-López A, de Jonge H, de Waal PP, Hong WS, Schulz RW 2010 Molecular cloning and functional characterization of a zebrafish nuclear progesterone receptor. *Biology of Reproduction* **82** 171-181.

de Waal PP, Wang DS, Nijenhuis WA, Schulz RW, Bogerd J 2008 Functional characterization and expression analysis of the androgen receptor in zebrafish (*Danio rerio*) testis. *Reproduction* **136** 225-234.

Dépêche J, Sire O 1982 *In vitro* metabolism of progesterone and 17α-

hydroxyprogesterone in the testis of the rainbow trout, *Salmo gairdneri* Rich., at different stages of spermatogenesis. *Reproduction Nutrition Development* **22** 427-438.

Evans RM 1988 The steroid and thyroid hormone receptor superfamily. *Science* **240** 889-895.

Fernandes MS, Brosens JJ, Gellersen B 2008 Honey, we need to talk about the membrane progestin receptors. *Steroids* **73** 942-952.

Galena HJ, Pillai AK, Terner C 1974 Progesterone and androgen receptors in non-flagellate germ cells of the rat testis. *Journal of Endocrinology* **63** 223-237.

González-Morán MG, Guerra-Araiza C, Campos MG, Camacho-Arroyo I 2008 Histological and sex steroid hormone receptor changes in testes of immature, mature, and aged chickens. *Domestic Animal Endocrinology* **35** 371-379.

Graham JD, Clarke CL 1997 Physiological action of progesterone in target tissues. *Endocrine Review* **18** 502-519.

Hanna RN, Zhu Y 2009 Expression of membrane progestin receptors in zebrafish (*Danio rerio*) oocytes, testis and pituitary. *General and Comparative Endocrinology* **161** 153-157.

Hanna RN, Daly SC, Pang Y, Anglade I, Kah O, Thomas P, Zhu Y 2010 Characterization and expression of the nuclear progestin receptor in zebrafish gonads and brain. *Biology of Reproduction* **82** 112-122.

Hoegg S, Brinkmann H, Taylor JS, Meyer A 2004 Phylogenetic timing of the fish-specific genome duplication correlates with the diversification of teleost fish. *Journal of Molecular Evolution* **59** 190-203.

Ikeuchi T, Todo T, Kobayashi T, Nagahama Y 2002 A novel progesterone receptor subtype in the Japanese eel, *Anguilla japonica*. *FEBS Letters* **510** 77-82.

Kohler C, Riesenbeck A, Hoffmann B 2007 Age-dependent expression and localization of the progesterone receptor in the boar testis. *Reproduction in Domestic Animals* **42** 1-5.

Kusakabe M, Kobayashi T, Todo T, Lokman PM, Nagahama Y, Young G 2002 Molecular cloning and expression during spermatogenesis of a cDNA encoding testicular 11β-hydroxylase (P45011β) in rainbow trout (*Oncorhynchus mykiss*). *Molecular Reproduction and Development* **62** 456-469.

Kusakabe M, Nakamura I, Evans J, Swanson P, Young G 2006 Changes in mRNAs encoding steroidogenic acute regulatory protein, steroidogenic enzymes and receptors for gonadotropins during spermatogenesis in rainbow trout testes. *Journal of Endocrinology* **189** 541-554.

Leal MC, Cardoso ER, Nóbrega RH, Batlouni SR, Bogerd J, França LR, Schulz RW 2009 Histological and stereological evaluation of zebrafish (*Danio rerio*) spermatogenesis with an emphasis on spermatogonial generations. *Biology of Reproduction* **81** 177-187.

Luetjens CM, Didolkar A, Kliesch S, Paulus W, Jeibmann A, Bocker W, Nieschlag E, Simoni M 2006 Tissue expression of the nuclear progesterone receptor in male non-human primates and men. *Journal of Endocrinology* **189** 529-539.

Mayer I, Lundqvist H, Berglund I, Schmitz M, Schulz R, Borg B 1990 Seasonal endocrine changes in Baltic salmon, *Salmo salar*, immature parr and mature male parr. I. Plasma levels of five androgens, 17-hydroxy-20*β*-dihydroprogesterone, and 17*β*-estradiol. *Canadian journal of zoology* **68** 1360-1365.

Miura C, Ohta T, Ozaki Y, Tanaka H, Mirua T 2009 Trypsin is a multifunctional factor in spermatogenesis. *Proceedings of the National Academy of Sciences of the United States of America* **106** 20972-20977.

Miura T, Higuchi M, Ozaki Y, Ohta T, Miura C 2006 Progestin is an essential factor for the initiation of the meiosis in spermatogenetic cells of the eel. *Proceedings of the National Academy of Sciences of the United States of America* **103** 7333-7338.

Miura T, Yamauchi K, Takahashi H, Nagahama Y 1992 The role of hormones in the acquisition of sperm motility in salmonid fish. *Journal of Experimental Zoology* **261** 359-363.

Ohno S 1970 Evolution of gene duplication. New York: Springer Verlag.

Ogino Y, Katoh H, Yamada G 2004 Androgen dependent development of a modified anal fin, gonopodium, as a model to understand the mechanism of secondary sexual character expression in vertebrates. *FEBS Letters* **575** 119-126.

Ogino Y, Katoh H, Kuraku S, Yamada G 2009 Evolutionary history and functional characterization of androgen receptor genes in jawed vertebrates. *Endocrinology* **150** 5415-5427.

Ozaki Y, Higuchi M, Miura C, Yamaguchi S, Tozawa Y, Miura T 2006 Roles of 11*β*-hydroxysteroid deheydrogenase in fish spermatogenesis. *Endocrinology* **147** 5139-5146.

Schulz RW, Andersson E, Taranger GL 2006 Photoperiod manipulation can stimulate or inhibit pubertal testis maturation in Atlantic salmon (*Salmo salar*). *Animal Reproduction* **3** 121-126.

Schulz RW, de França LR, Lareyre JJ, LeGac F, Chiarini-Garciac H, Nobrega RH, Miura T 2010 Spermatogenesis in fish. *General and Comparative Endocrinology* **165** 390-411.

Schulz RW, Menting S, Bogerd J, de Françac LR, Vilela DAR, Godinho HP 2005 Sertoli cell proliferation in the adult testis: evidence from two fish species belonging to different orders. *Biology of Reproduction* **73** 891-898.

Schulz RW, van der Corput L, Janssen-Dommerholt C, Goos HJTh 1994 Sexual steroids during puberty in male African catfish (*Clarias gariepinus*): serum levels and gonadotropin-stimulated testicular secretion in vitro. *Journal of Comparative Physiology. B, Biochemical, Systemic, and Environmental Physiology* **164** 195-205.

Scott AP, Sumpter JP 1989 Seasonal variations in testicular germ cell stages and in plasma concentrations of sex steroids in male rainbow trout (*Salmo gairdneri*) maturing at 2 years old. *General and Comparative Endocrinology* **73** 46-58.

Shah C, Modi D, Sachdeva G, Gadkar S, Puri C 2005 Coexistence of intracellular and membrane-bound progesterone receptors in human testis. *The Journal of*

Clinical Endocrinology and Metabolism **90** 474-483.

Sirivaidyapong S, Bevers MM, Gadella BM, Colenbrander B 2001 Induction of the acrosome reaction in dog sperm cells is dependent on epididymal maturation: the generation of a functional progesterone receptor is involved. *Molecular Reproduction and Development* **58** 451-459.

Thornton JW 2001 Evolution of vertebrate steroid receptors from an ancestral estrogen receptor by ligand exploitation and serial genome expansions. *Proceedings of the National Academy of Sciences of the United States of America* **98** 5671-5676.

Todo T, Ikeuchi T, Kobayashi T, Kajiura-Kobayashi H, Suzuki K, Yoshikuni M, Yamauchi K, Nagahama Y 2000 Characterization of a testicular 17*α*,20*β*-dihydroxy-4-pregnen-3-one (a spermiation-inducing steroid in fish) receptor from a teleost, Japanese eel (*Anguilla japonica*). *FEBS Letters* **465** 12-17.

Tubbs C, Thomas P 2008 Functional characteristics of membrane progestin receptor alpha (mPR*α*) subtypes: a review with new data showing mPR*α* expression in seatrout sperm and its association with sperm motility. *Steroids* **73** 935-941.

Ueda H, Kanbegawa A, Nagahama Y 1985 Involvement of gonadotrophin and steroid hormones in spermiation in the amago salmon, *Oncorhynchus rhodurus*, and goldfish, *Carassius auratus*. *General and Comparative Endocrinology* **59** 24-30.

Umesono K, Evans RM 1989 Determinants of target gene specificity for steroid/thyroid hormone receptors. *Cell* **57** 1139-1146.

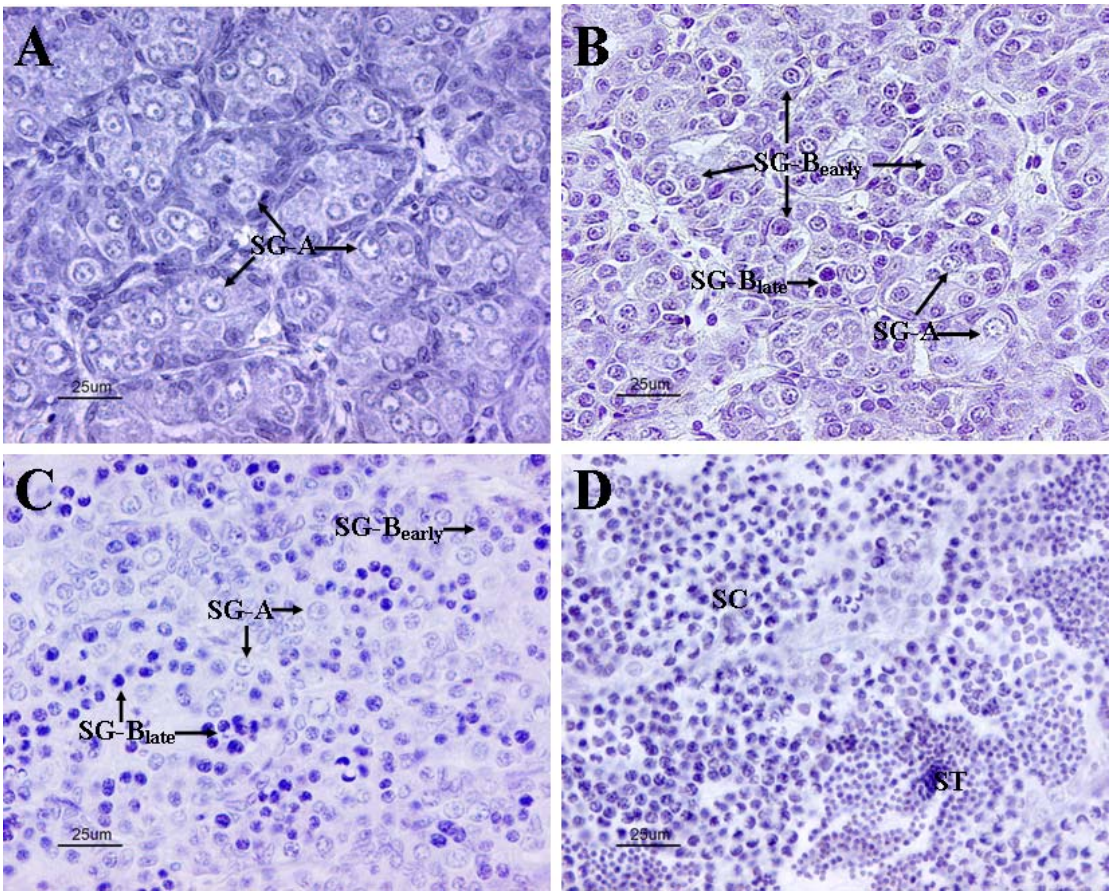
Vischer HF, Teves AC, Ackermans JC, van Dijk W, Schulz RW, Bogerd J 2003 Cloning and spatiotemporal expression of the follicle-stimulating hormone β subunit complementary DNA in the African catfish (*Clarias gariepinus*). *Biology of Reproduction* **68** 1324-1332.

Vizziano D, LeGac F, Fostier A 1996 Effect of 17*β*-estradiol, testosterone, and 11-ketotestosterone on 17*α*,20*β*-dihydroxy-4-pregnen-3-one production in the rainbow trout testis. *General and Comparative Endocrinology* **104** 179-188.

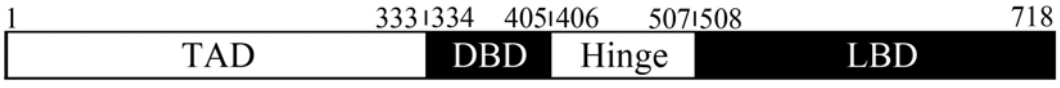
Wang L, Sanyal S, Oh DY, Kim J, Ju JW, Song K, Kim JW, Kwon HB, Choi H 2004 Molecular cloning and characterization of an amphibian progesterone receptor from *Rana dybowskii*. *General and Comparative Endocrinology* **135** 142-149.

Wittbrodt J, Meyer A, Schartl M 1998 More genes in fish? *Bioessays: News and Reviews in Molecular, Cellular and Developmental Biology* **20** 511-515

Supplemental information

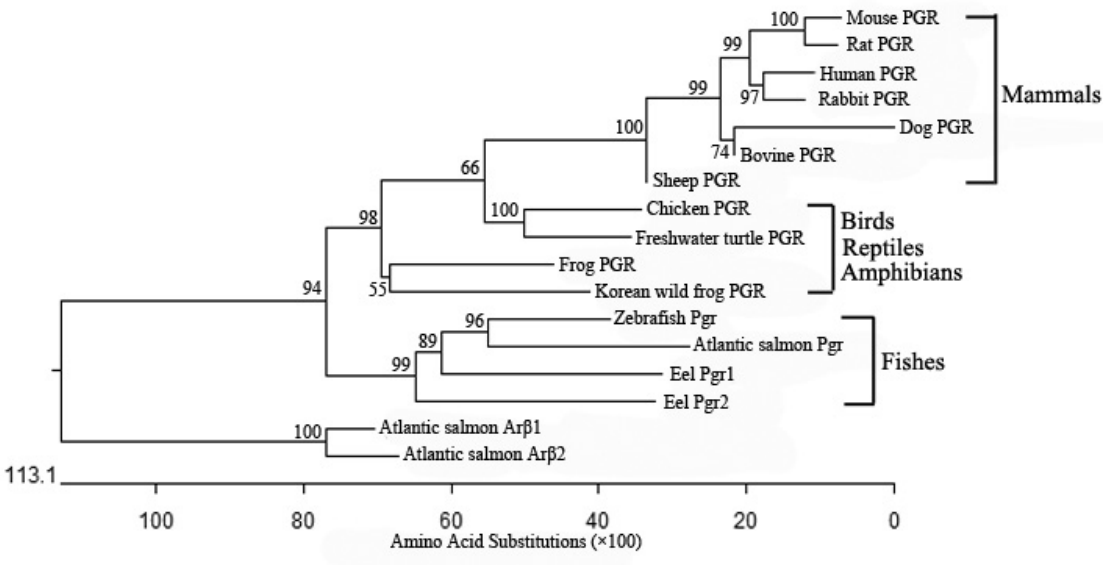


Supplemental Figure 1 Hematoxylin-stained, 5 μm sections from Atlantic salmon testis showing different stages of germ cell development. Labeled structures are type A spermatogonia (SG-A); early type B spermatogonia (SG-B_{early}); late type B spermatogonia (SG-B_{late}); spermatocyte (SC), and spermatid (ST).



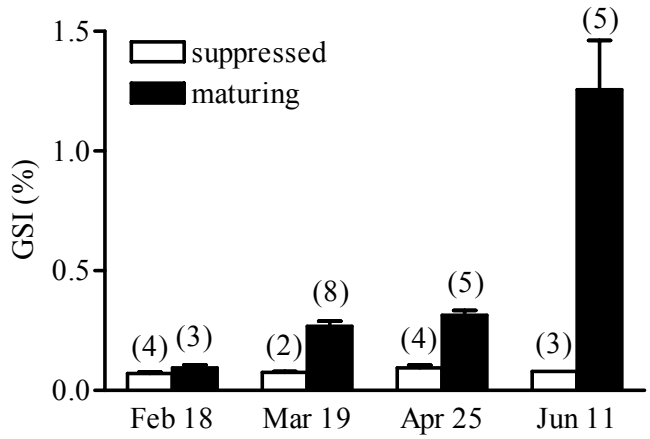
Supplemental Figure 2 Domains of the salmon progesterone receptor

Schematic representation of the salmon Pgr and its domains: TAD, transactivation domain; DBD, DNA-binding domain; Hinge, hinge domain; LBD, ligand-binding domain. Previously, the TAD, DBD, hinge and LBD domains were named A/B, C, D and E/F domains, respectively. The numbers above each box refer to the amino acid positions delimiting the domains.

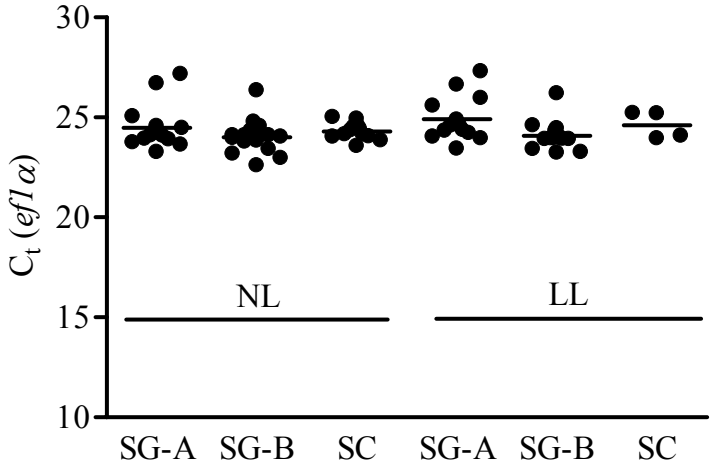


Supplemental Figure 3 Phylogenetic tree of PGRs

The phylogenetic tree was constructed by the neighbor-joining method using the MegAlign program (Lasergene software package; DNASTAR Inc.), including only sequences where progesterone binding had been demonstrated experimentally, and rooted by two types of salmon androgen receptor (unpublished data). The horizontal distances to the branching points are proportional to the number of amino acid substitutions. The numbers next to the branches indicate bootstrap values from 1000 replicates.



Supplemental Figure 4 Gonado-somatic index (GSI) of male Atlantic salmon exposed to constant light (LL) starting 1st of February. Based on the testicular histology, males sampled at the indicated month were assigned to two sub-groups (immature and maturing testis). The number of animals per sub-group is indicated between brackets above each column.



Supplemental Figure 5 C_t values of *ef1a* mRNA during early spermatogenesis. Males were exposed to additional continuous light (LL) or to natural light (NL) from February 1. One-way ANOVA was used to compare groups formed on the basis of photoperiod and stage of germ cell development; significant differences were not found. SG-A, type A spermatogonia; SG-B, type B spermatogonia; SC, spermatocytes.

Supplemental Table 1 Primers used in this study

Primer	Nucleotide sequence (5'→3')
2699	GGGGATGAAGCCTCTGGCTGTCATTA
2700[a]	CTTGGYTGAGGATRAGATCTGGTGCAAAGTAC
2720	TCCTCACATGTGGAAGCTGCAAAGTGTTCTTTAAACG
2721	CGGTCGAAATGACTGCATTGTGGATAAGATACGG
2722	GTGTCATCTGATCGTTGATGTGGAGGCTACGGAAC
2723	GGCAGGGACTTGGACCACCGGACG
2767	GCTCCTGATCTTATCCTTAGCCAGGATCGTATGAGGAGAT
2768	CAGTTCATCCCTCAAGAGTTCACCAGCCTCCAAG
2794	TTGCCACCATGGACACGGCGAACACGTTGAGCT
2796	TGTGATTGGTCACTTGGCGTGGAAAGGAG
2831	ATTGTGGATAAGATACGGAG
2832	CCCAGCCTGGTAGCACTTC
3168[b]	T7Rpps-TCTTGACCATGCCGGCCAG
3198[c]	T3Rpps-CCCTTGTCAGCCCTGGGG

[a]Primer 2700 is a degenerate primer; Y = T or C and R = G or A.

[b]Primer 3168 contains the T7 RNA polymerase promoter sequence (underlined) at its 5'-end (T7Rpps; 5'-CCGGGGGGTGTAATACGACTCACTATAGGG-3')

[c]Primer 3198 contains the T3 RNA polymerase promoter sequence (underlined) at its 5'-end (T3Rpps; 5'-GGGCGGGTGTTATTAACCCTCACTAAAGGG-3')

Supplemental Table 2 Percentage amino acid identity of the salmon Pgr compared with PGRs from other species.

	Full length	TAD	DBD	Hinge	LBD
Zebrafish Pgr	57.2	22.2	97.2	70.9	85.3
Eel Pgr1	52.5	21.3	95.8	64.8	83.7
Eel Pgr2	51.5	19.4	91.7	59.3	83.7
Human PGR	38.6	12.9	90.3	33.7	67.4
Dog PGR	38.2	11.4	90.3	32.6	67.4
Chicken PGR	37.9	10.8	90.3	35.3	65.8
Frog PGR	40.4	13.2	88.9	40.2	68.4
Mouse PGR	39.0	13.2	90.3	32.6	66.3
Rat PGR	38.6	13.2	90.3	33.7	67.4
Korean wild frog PGR	37.0	10.5	83.3	38.6	64.7
Rabbit PGR	38.2	12.3	90.3	31.7	67.4
Sheep PGR	62.9	26.7	90.3	34.9	67.4
Bovine PGR	52.7	15.0	90.3	36.0	67.4
Freshwater turtle PGR	39.7	12.6	90.3	40.7	66.8

The amino acid sequences of the different progesterone receptors were gathered from the GenBank database (see below) and domain by domain homology analysis with salmon Pgr was performed using MegAlign/DNASTAR software. See Figure 1 for a key to the domain nomenclature.

Zebrafish (*Danio rerio*) (FJ409244), Japanese eel (*Anguilla japonica*) (receptor 1, BAA89539; and 2 AB028024), human (*Homo sapiens*) (M15716), rabbit (*Oryctolagus cuniculus*) (M14547), bovine (*Bos Taurus*) (AY656812), dog (*Canis lupus familiaris*) (AF177470), sheep (*Ovis aries*) (Z66555), rat (*Rattus norvegicus*) (L16922), mouse (*Mus musculus*) (M68915), frog (*Xenopus laevis*) (AF279335), chicken (*Gallus gallus*) (P07812), Korean wild frog (*Rana dybowskii*) (AF431813), freshwater turtle (*Pseudemys nelsoni*) (AB301062). Domain by domain homology analysis with the zebrafish Pgr was performed using MegAlign/DNASTAR software. See supplemental figure 1 for key to the specific domains nomenclature.

Chapter 5

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Cloning, pharmacological characterization and expression analysis of Atlantic cod (*Gadus morhua*, L.) nuclear progesterone receptor

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Abstract

To better understand the role(s) of progesterone during spermatogenesis in fish, we cloned the nuclear progesterone receptor (Pgr) of Atlantic cod. The open-reading frame of the *pgr* consists of 2076 bp, coding for a 691 amino acids-long protein that shows the highest similarity with other piscine Pgr proteins. Functional characterization of the receptor expressed in mammalian cells revealed that the cod Pgr exhibited progesterone-specific, dose-dependent induction of reporter gene expression, with 17*α*,20*β*-dihydroxy-4-pregnen-3-one (DHP), a typical piscine progesterone, showing the highest potency in activating the receptor. During ontogenic development, the *pgr* mRNA was undetectable in embryo's 24 h after fertilization, but became detectable 4 days after fertilization. During the larval stage, expression levels increased steadily with the development of the larvae. In adult fish, *pgr* was predominantly expressed in the gonads of both sexes. During the onset of puberty, testicular *pgr* transcript levels started to increase during massive spermatogonial proliferation, and peaked when spermiation started in pre-spawning fish. *In situ* hybridization studies using testis tissue during the rapid growth phase containing all germ cell stages indicated that in cod, *pgr* mRNA is predominantly located in Sertoli cells in contact with rapidly proliferating spermatogonia. Taken together, our data suggests that the Pgr is involved in mediating DHP-stimulated, mitotic expansion of the spermatogonial population, and in processes associated with the spermiation/spawning period in Atlantic cod.

Introduction

In mammals, the role of progesterone signaling in a variety of reproductive processes like menstruation, mammary gland development, establishment and maintenance of pregnancy, and lactation is well established (Clarke and Sutherland 1990). Moreover, data is accumulating that progesterone also has a role in male reproductive events (Walton *et al*. 2006). In teleost fish, for example, 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 the induction of sperm maturation (Miura *et al*. 1992, 1995), next to their roles during the resumption of meiosis in final oocyte maturation (Nagahama 1997). Recent evidence indicates that progestin possesses multiple regulatory functions at early stages of fish spermatogenesis. For example, in Japanese eel (*Anguilla japonica*) DHP has the ability to induce spermatogonial DNA synthesis and the initiation of meiosis, by inducing expression of the short form of 11*β*-hydroxysteroid dehydrogenase (11*β*-HSD) in Leydig cells and of trypsinogen in Sertoli cells (Miura *et al*. 2006; Miura *et al*. 2009).

Many of the biological actions of progestins are mediated through the progesterone receptor (PGR), a member of the nuclear receptor superfamily of ligand-activated transcription factors (Evans 1988). The gene encoding this receptor arose early in vertebrate evolution via a series of duplications of an ancestral estrogen receptor

(Thornton 2001). The reproductive functions of the PGR have been well studied in mammalian models (Conneely *et al*. 2002). However, less information is available as regards the specific biological functions of Pgr in teleost fish, the largest and most diverse group of vertebrates. Our previous research indicated that also in zebrafish (*Danio rerio*), DHP mediates Pgr induction of 11β-HSD activity in adult testis tissue (Chen *et al*. 2010). In addition, ongoing research in Atlantic salmon (*Salmo salar* L.) suggests a role of Pgr in mediating DHP-stimulated, early steps of spermatogenesis, such as the differentiation of late type B spermatogonia and perhaps also the entry into meiosis (unpublished data).

The Atlantic cod (*Gadus morhua*, L.), a member of the order of Gadiformes, is an economically important marine fish in the Northern hemisphere (Norberg *et al*. 2004). Recent research on spermatogenesis in this species revealed a novel mode of cystic germ cell development that occurs in a specific spatio-temporal organization: testis tissue is composed of several lobes, in which undifferentiated spermatogonia are located in the periphery of each lobe, while increasingly advanced stages of germ cell development are found in a maturational gradient towards the common collecting duct. Spermatogenesis in cod therefore represents an intermediate form between restricted and unrestricted spermatogonial distribution (Almeida *et al*. 2008).

To study the role of Pgr during spermatogenesis in teleost fish from another order than Anguilliformes, Cypriniformes or Salmoniformes, we set out to clone the cod *pgr* cDNA. After pharmacological characterization of the cod Pgr, we examined the expression profile of the *pgr* mRNA during ontogenesis as well as in different adult tissues. Thereafter, we determined the cellular localization of the *pgr* mRNA in cod testis by *in situ* hybridization. Finally, we analyzed changes in testicular *pgr* expression during the onset of puberty (verified individually by histological analysis of the testis).

Material and methods

Cod *pgr* cDNA cloning

To isolate the cod *pgr* cDNA, we used a set of degenerate primers (primers 2197 and 2198; Supplementary Table 1) in a PCR on random-primed cod brain cDNA, and obtained a ~730 bp PCR product that was gel purified and cloned. Four clones were sequenced, all providing the same partial *pgr* cDNA sequence, while no evidence for the presence of a second cod *pgr* cDNA sequence was found with this primer set.

To isolate the 5'- and 3'-ends of the cod *pgr* cDNA, gene-specific primers (primers 2228 and 2235; Supplemental Table 1) based on the consensus nucleotide sequence of the 4 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 (primers 2229 and 2236; see Supplemental Table 1), respectively, in combination with a nested universal primer (NUP). Both the UPM and NUP were supplied with the SMART RACE cDNA amplification kit (Clontech, Mountain View, CA, USA). All RACE reactions were carried out according to the manufacturer's instructions in a Perkin-Elmer 2400

Thermal Cycler (Applied Biosystems, Foster City, CA, USA) using Advantage 2 polymerase (Clontech). RACE products were subcloned into pcDNA3.1/V5-His TOPO vector (Invitrogen, Breda, The Netherlands).

Combining the partially overlapping sequences of the 5'- and 3'-RACE products yielded a full-length cod *pgr* cDNA sequence with 2076 nucleotides in the open-reading frame. The open-reading frame was PCR amplified using primers 2259 and 2260, and subcloned in the correct orientation into the pcDNA3.1/V5-His-TOPO expression vector. Sequence analysis of several clones revealed identical *pgr* sequences as those obtained in the 5'- and 3'-RACE clones, and the sequence of this cod *pgr* open-reading frame was deposited at GenBank with accession number EU625299.

Transactivation assays for cod 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 medium (without phenol red) supplemented with 10% v/v fetal bovine serum (FBS), glutamine, and penicillin/streptomycin (Gibco, Breda, The Netherlands) at 37°C in a 5% CO_2 incubator. After 24 h, the cells were co-transfected with 1 µg of the cod *pgr* expression plasmid and 7 µg of pGL3-MMTV-Luc plasmid using a standard calcium phosphate precipitation method. 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 with final concentrations ranging between 0.1 nM and 10 µM (n=2 per condition tested). After incubation at 37°C for 24-36 h, the cells were harvested in lysis mix (100 mM potassium phosphate buffer pH 7.7, 1% v/v Triton X-100 [Sigma-Aldrich], 15% v/v glycerol, and 2 mM dithiotreitol [DTT]) and stored at -80°C. Luciferase activity was determined by adding an equal volume of substrate mix (100 mM potassium phosphate buffer pH 7.7, 250 mM D-luciferin [Invitrogen], 1 mM DTT, 2 mM ATP [Roche, Woerden, The Netherlands] and 15 mM magnesium sulfate [Promega, Leiden, The Netherlands]) to thawed samples and luminescence was measured in a Packard TopCount NXT luminometer (Perkin Elmer Life Sciences, Meriden, CT, U.S.A.). Each compound was tested in three independent experiments using cells from different transfections.

The following steroids were used in this study: DHP, 20*β*-S, progesterone (P4), 17*α*-hydroxy-4-pregnen-3-one (17*α*(OH)P4), testosterone (T), 11-ketotestosterone (11-KT), 17*β*-estradiol (E_2), cortisol, and the synthetic progestin promegestone (R5020). All steroids were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands) except for R5020 that was obtained from Perkin Elmer (Waltham, MA, USA).

Cod *pgr* mRNA expression during ontogenic development and in adult tissue

Total RNA was isolated from a pool of the following materials: embryos 24 hours and 4 days after fertilization; larvae of 5 to 6 mm total length, just before first feeding. Total RNA was extracted from individual larvae, after head removal, from 6.3 mm to

57 mm full-length (n=2-21); the sex of the larvae was unknown, as a genetic sex marker is not yet available for Atlantic cod. In adult cod, total RNA was isolated from head kidney, gill, spleen, heart, intestine, liver, muscle, and gonad of three males and three females.

To examine a variation in the *pgr* mRNA expression with the changes in testis growth and spermatogenic activity during the onset of puberty in cod, we measured the *pgr* mRNA levels from testis tissue of animals under pubertal development. The maintenance and sampling of Atlantic cod were described in a previous report (Almeida *et al*. 2008). Briefly, male Atlantic cod (Norwegian Coastal Cod) were reared in light-proof 7 m^3 seawater tanks (n = 175/tank) at the Institute of Marine Research, Austevoll Research Station, Norway (60°N). The tanks were supplied with artificial illumination simulating the local natural photoperiod during 18 months (July 2004 to November 2005; NL) or 6 months with constant light (from July 2004 until winter solstice) followed by 11 month of simulated natural light (LL-NL). Samples were collected monthly in the NL group and after the light change in the LL-NL group (i.e. only in fish under NL exposure, when testis development took place) . A fragment of testis tissue was dissected, wrapped in aluminium foil and immediately snap-frozen by immersion in liquid nitrogen, before storage at -80°C. Other fragments were fixed and later embedded in resin or paraffin for histological classification of the gonad and for immunohistochemistry, respectively. All fish were treated and euthanized according to Norwegian National Legislation for Laboratory Animals.

All RNA extractions were performed by the acid phenol-guanidine thiocyanate method after tissue homogenization in the FastPrep tube containing Lysing Matrix D ceramics beads (MP Biomedicals, Solon, OH, USA). DNase treatment (Turbo DNA-free, Ambion/Applied Biosystems, Oslo, Norway) was applied to 10 µg of total RNA before reverse transcription. Random-primed cDNA was synthesized from 500 ng RNA using the Reverse Transcriptase Core Kit (RT-RTCK-05, Eurogentec, Belgium) according to the manufacturer's instructions (Mitteholzer *et al*. 2007).

To detect cod *pgr* using quantitative real-time PCR (qPCR), primers 3068 and 3069 (Supplemental Table 1) were designed using PrimerExpress software (Applied Biosystems, Foster City). Atlantic cod *elongation factor 1α* (*ef1α*) was used as endogenous control as described in detail previously (Mitteholzer *et al*. 2007); no significant differences in *ef1α* expression levels were found in the testes samples analyzed (Supplemental Figure 3). All qPCRs were performed in 20 µl reactions and C_t values were determined in a 7900HT Real-Time PCR System (Applied Biosystems) using default settings. Differences in gene expression were calculated with the $\Delta\Delta_{Ct}$ method, as described by Bogerd *et al*. (2001).

The samples, in which cod *pgr* expression during ontogenesis were measured, were normalized to *ef1α* and calibrated with the mean *pgr* mRNA expression in embryos (4 dpf), which was set to 1 (see Fig. 2A). Expression levels in the different cod tissues were normalized to *ef1α* and calibrated with the mean *pgr* mRNA expression in immature testis, which was set to 1 (see Fig. 2B). Expression levels in testis tissue are presented in two ways: relative to Atlantic cod *ef1a* mRNA, which was set to 1 (see Fig. 2C), and relative to *ef1a* mRNA, but also corrected for RNA yield (µg RNA

recovered per mg tissue extracted), testis mass and body weight as described by Kusakabe *et al*. (2006), and then shown relative to the tissue at immature stage, which was set to 1 (see Fig. 2D).

Cellular localization of *pgr* expression in cod testis

Localization of receptor expression by *in situ* hybridization was done as described previously (Chen *et al*. 2010), using 10 μm cryo-sections prepared from paraformaldehyde-fixed testis tissue from fish sampled during the onset of spermatogenesis. Specific primers (3197 and 3130; Supplementary Table 1) were designed to PCR amplify a cod *pgr* cDNA fragment for sense and antisense digoxigenin-labeled cRNA probe synthesis.

To identify Sertoli cell cytoplasm and to differentiate it from germ cells within a cyst, we used an antibody against vimentin, an intermediate filament of the Sertoli cell cytoskeleton (reviewed in Vogl *et al*. 2008). The immunocytochemistry protocol was modified from a previous report (Leal *et al*. 2009). Briefly, sections of 4% paraformaldehyde-fixed and paraffin-embedded cod testis were dried overnight at 37°C. After deparaffinization and hydration, the sections were subjected to antigen retrieval by boiling for 10 min in 10mM Citrate buffer pH 6.0 and peroxidase blocking (immersion in 0.3% H_2O_2 in PBS for 10 min) followed by incubation with mouse IgM anti-human vimentin (non-hematopoietic LN6; 1:200 dilution, Biogenex, San Ramon, CA, USA) at 4°C overnight. Thereafter, the sections were incubated with biotinylated goat anti-mouse IgM (1:200 dilution) for 1 h at room temperature, and with avidin-biotin complex (ABC Kit- Vector Laboratories) for another hour. DAB (3,3'-diaminobenzidine tetrahydrochloride, Dako, Glostrup, Denmark) substrate development was done for approximately 2 min. Nuclei were counterstained with 5% Mayer hematoxylin for 45 sec. As positive control, the procedure was applied to rat testis sections, and as negative control the primary antibody was replaced by the same concentration of normal mouse IgM, using both cod and rat testis sections.

Results

Isolation and sequence analysis of cod *pgr* cDNA

The open-reading frame (ORF) of the cod *pgr* consisted of 2076 nucleotides (GenBank accession number: EU625299), encoding a protein of 691 amino acids (Supplemental Fig. 1). Comparison of the deduced amino acid sequence of the cod Pgr with receptors from other species is shown in Supplemental Table 2. The overall homology analysis indicated that cod Pgr is more similar to teleost (59-67%) than to tetrapod (33-52%) PGR forms. As a member of the nuclear receptor family, the cod Pgr amino acid sequence could be subdivided into 4 domains. The N-terminal transactivation domain (TAD) is a strong regulator of transcription. However, its sequence showed low homology (8-30%) with other PGRs, and is divergent in length and primary sequences among vertebrates. On the other hand, the putative DNA-binding domain (DBD) and ligand-binding domain (LBD) are highly conserved (DBD, 84-100%; LBD, 67-88%) among all vertebrate PGRs studied to date.

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 (Supplemental Fig. 2). One consisted of fish Pgrs, a second clade contained avian, reptilian, and amphibian PGRs, and the last grouped mammalian PGRs. Within the teleost Pgr group, 99% bootstrap support distinguished a zebrafish lineage from the eel group.

Steroid-specific transactivation of the cod Pgr

To determine the steroid-dependent transactivation properties of the cod 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 cod *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), with the former presenting the lowest EC_{50} value (172.8±1.2 nM). Also at a fixed concentration of 1 μM, DHP and 20*β*-S were the most potent inducers of luciferase activity (30-fold and 15-fold above control, respectively; Fig. 1B). The other three progesterone-related hormones tested elicited increases of luciferase activity only at a concentration of 10 μM, while other steroid hormones assayed (T, 11-KT, E_2, or cortisol) were ineffective at 10 μM.

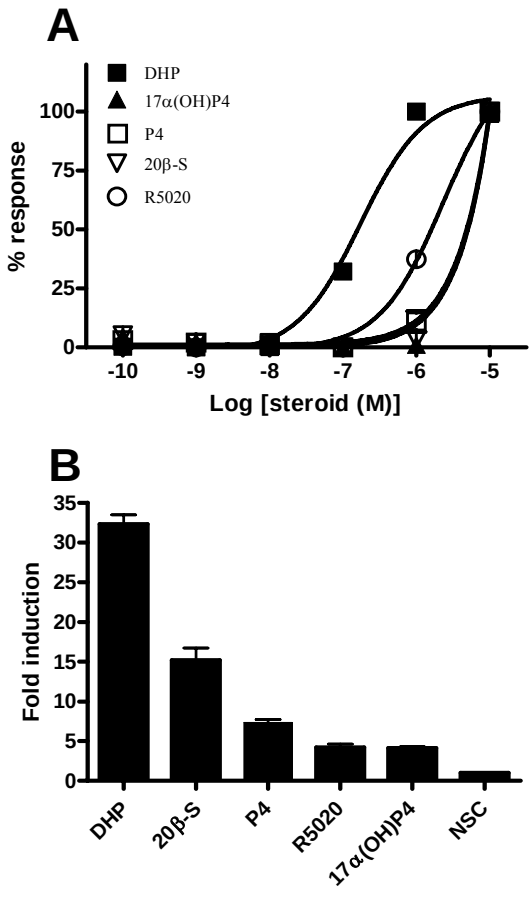

Figure 1 Ligand-induced transactivation properties of the Atlantic cod Pgr, after transient co-transfection of HEK 293T cells with the cod *pgr* expression vector construct and the pGL3-MMTV-Luc reporter construct. (A) Transfected cells were incubated with increasing concentrations (from 0.1 nM to 10 μM) of various progesterones. Percentage (%) response: values are given relative to the maximal amount of luciferase activity for each condition. Each point represents the mean ± SEM 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 μM of the steroids indicated. Data are expressed as the ratio of steroid versus NSC (no steroid control). Each column represents the mean of three independent experiments, with the vertical bar representing the SEM.

Expression profiles of cod *pgr* mRNA during ontogenic development and in adult tissues

Real-time, quantitative PCR analysis of several organs from male and female cod showed that *pgr* mRNA was predominantly expressed in the gonad of both sexes. Besides, *pgr* mRNA were also found in the liver of females and in the intestine of both sexes, while *pgr* mRNA was scarce or undetectable in muscle, spleen, heart, gill and head kidney (Fig. 2A).

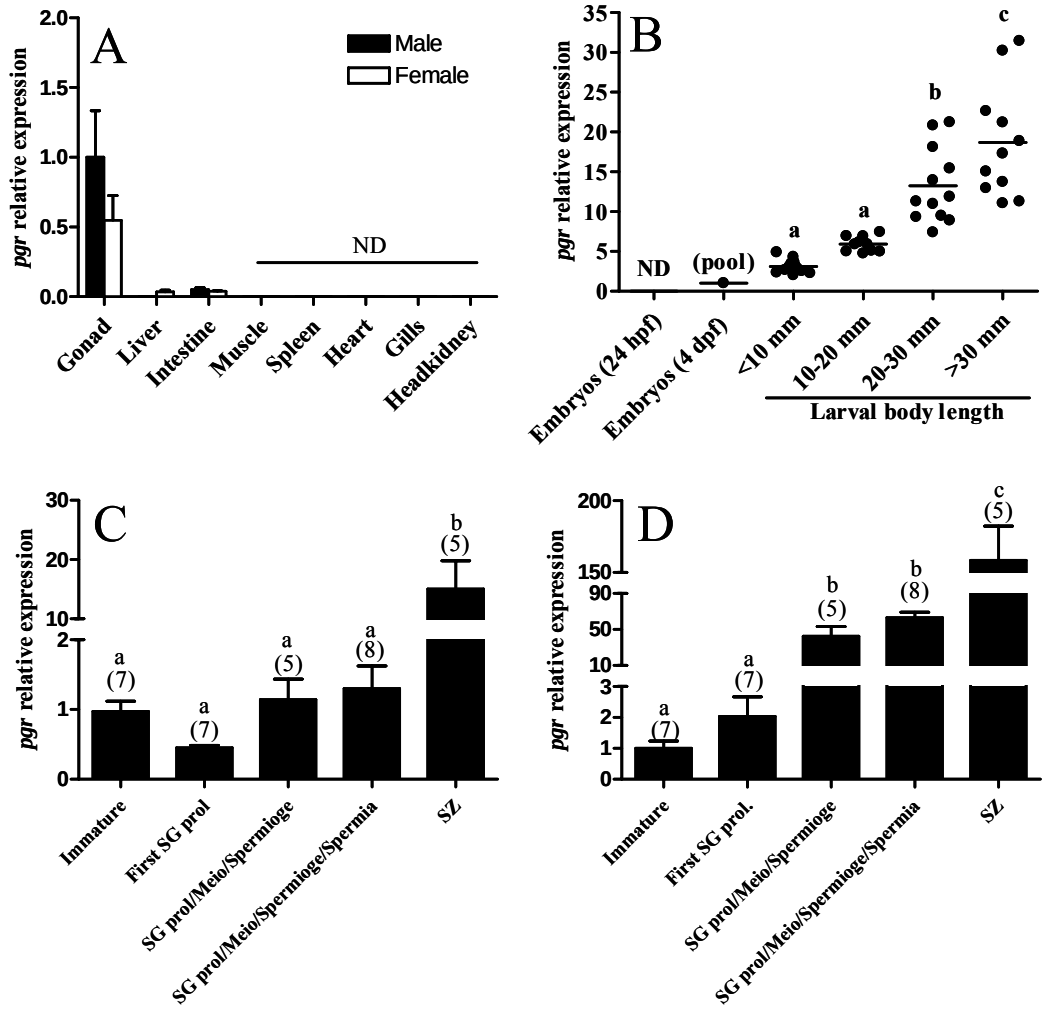

Figure 2 Expression of cod *pgr* mRNA.

(A) Relative expression *of pgr* mRNA levels in adult organs of male and female cod (n=3). Values represent mean ± SEM relative to *pgr* mRNA levels in testis. ND, not detectable. (B) Relative expression of *pgr* mRNA levels in cod embryos throughout early development. Values represent mean ± SEM relative to *pgr* mRNA levels in embryos 4 days post fertilization (4 dpf). ND, not detectable; 24 hpf, 24 hours post fertilization. (C, D) Relative expression of *pgr* mRNA levels in testis during one cycle of spermatogenesis. Values represent mean ± SEM relative to *pgr* mRNA levels in immature testis. Males were assigned to groups based on the testis histology, with abbreviations indicating: SG Prol, spermatogonia proliferation; Meio, meiosis; Spermioge, spermiogenesis; Spermia, spermiation; SZ, spermatozoa. The number of animals per group is indicated between brackets above each column. Bars marked with different letters are significantly different from each other (P<0.05). ND, not detectable.

Analysis of cod embryos showed that the *pgr* mRNA was undetectable 24 h after fertilization, and became detectable in embryos 4 days after fertilization. During the larval stage, the lowest *pgr* expression was found in larvae of <10 mm length, and increased steadily with the development of the larvae (samples were up to 57mm) (Fig. 2B).

As described in a previous report (Almeida *et al*. 2008), pre-pubertal males kept under normal light conditions are progressively recruited into maturation in August and September and then undergo a testicular growth phase until February, during which spermatogenesis takes place, progressively filling the spermatogenic tubule with spermatozoa; testis weight increased 41-fold and reached almost 10% of the total body weight. When LL males were moved to NL in December, testicular weight gain reached maximum GSI values, which was similar to those in the NL group, during the following 5 months that was compressed in time compared to male in NL condition. When males from both NL and LL→NL were grouped according to the stage of germ cell development, the expression of testicular *pgr* mRNA was low but clearly detectable in immature testes. The levels did not change until spermiation started, when a significant up-regulation was recorded in testis tissue filled with spermatozoa (Fig. 2C). When RNA yield, testis mass and body weight were used to correct the expression level, the general expression pattern was similar, but the lowest mean of testicular *pgr* expression was observed in immature testes, and the levels started to increase significantly when testis were in full spermatogenesis - when meiosis and spermiogenesis were going on - and again later when the tubules were filled with free spermatozoa (pre-spawning) (Fig. 2D).

Cellular localization of *pgr* expression in cod testis

The mRNA of cod *pgr* was detected in the cytoplasm of Sertoli cells. Not all Sertoli cells showed the same intensity of *pgr* expression; instead, the expression varied with the stage of development of the germ cells in a given cyst (Fig. 3A, B). The most intense staining was observed in Sertoli cells in contact with rapidly proliferating type B spermatogonia, identified by the size of the cells. Some Sertoli cells associated with more developed germ cells, identified as primary spermatocytes, presented a weak staining while those in contact with type A spermatogonia were mostly negative. Immunohistochemical localization of vimentin, a cytoskeletal element of Sertoli cells, the cytoplasm of Sertoli cell is not only visible outlining the border of spermatogenic cysts (Fig. 3C), but also protrudes punctuated manner towards the lumen of spermatogenic cysts in between the germ cells in the cyst (Fig. 3D). The same pattern of Sertoli cell cytoplasmic staining was observed in the cysts positive for *pgr* mRNA *in situ* hybridization, as shown in Figure 3B. No staining was observed when sections were incubated with sense cRNA (Fig. 3A inset).

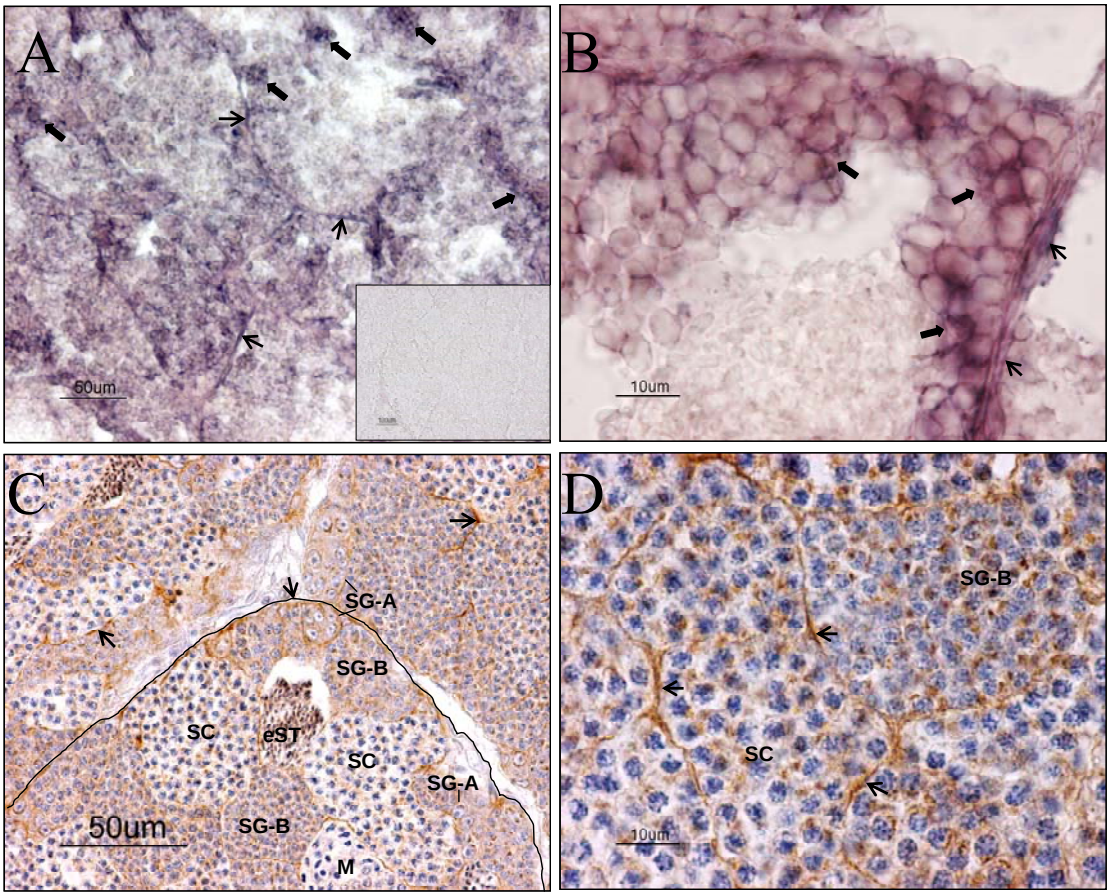

Figure 3 *In situ* hybridization analyses of *pgr* mRNA and immunohistochemistry of Vimentin in Atlancit cod testis. Low (A) and high (B) magnification of a cryosection of cod testis from animal during the onset of spermatogenesis, hybridized with the *pgr* antisense cRNA probe showing signal in the spermatogonial cysts at different developmental stage. Insert shows that the sense cRNA probe did not result in specific staining. Immunostaining of Vimentin of a paraffin-section of cod testis from animal during the onset of spermatogenesis at low (C) and high (D) magnification. Black arrows indicate positive cysts. Arrow points cytoplasm of Sertoli cells in the borders of spermatogenic cysts Cysts of: spermatocytes (SC); type A or B spermatogonia (SG-A, SG-B); elongated spermatids (eST). The black line borders a spermatogenic tubule, with different cysts inside.

Discussion

Piscine Pgrs have been previously cloned and functionally tested in species belonging to the orders Anguilliformes, Cypriniformes and Salmoniformes. In the present study, we cloned the open-reading frame of a cod *pgr* cDNA, which encodes a protein of 691 amino acids. Comparison of the deduced amino acid sequence of the cod Pgr with Pgrs from other species indicated that, similar to other members of the nuclear receptors family, the DBD and LBD domain are highly conserved (Evans, 1988). Moreover, the two zinc finger motifs, as well as the P box (GSCKV) and D box (AGRND) sequences were highly conserved in the DBD domain, suggesting these regions are functionally important for the Pgr to recognize the target gene sequences. Research on human Pgr indicated a proline rich motif in the N-terminal

domain, which is responsible for the interaction of the receptor with the c-Src family of tyrosine kinases (Boonyaratanakornkit *et al*. 2001). However, in cod Pgr, as well as in other piscine Pgrs, this motif was absent, so that a Pgr-mediated Mos/MAPK activation may not occur in teleost fish (Chen *et al*. 2010, Todo *et al*. 2000, Ikeuchi *et al*. 2002).

Due to the teleost-specific genome duplication, ray-finned fish can have two paralogous copies for many genes while only one ortholog is present in tetrapods (Wittbrodt *et al*. 1998). Two distinct *pgr* genes have been reported in the eel (Todo *et al*. 2000, Ikeuchi *et al*. 2002). However, the present study, in line with previous studies in zebrafish, medaka, Takefugu, Tetraodon, stickleback, and salmon (Chen *et al*. 2009; Hanna *et al*. 2010), did not provide evidence for the existence of additional *pgr*-like genes in the Atlantic cod. Two isoforms (forms A and B) encoded by the same gene but originating from different in-frame translational initiation codons have been reported for chicken and human progesterone receptor homologues (Conneely *et al*. 1987; Kastner *et al*. 1990). However, studies using PGR knockout mice indicated that only PGR-A is both necessary and sufficient to elicit the progesterone-dependent reproductive responses necessary for female fertility, while PGR-B is required to elicit normal proliferative responses of the mammary gland to progesterone (Conneely *et al*. 2002). Although we have found no evidence for it, the present study can not exclude the possible existence of other isoforms. However, considering the predominant expression of cod *pgr* in the gonads, we suggest that the cloned cod *pgr* may have important function(s) in the reproductive physiology of cod.

We demonstrated that the cod *pgr* cDNA codes for a functional Pgr, which is able to activate the transcription of a luciferase gene under control of the progesterone-regulated MMTV-LTR promoter (Truss and Beato, 1993). Moreover, transactivation was progesterone-specific and as in other teleost species, DHP was the most effective steroid in activating the Pgr. However, the EC_{50} value of DHP for cod (172 nM) Pgr was higher than for other fishes Pgr (0.12 nM in eel Pgr2, Ikeuchi *et al*. 2002; 8 nM in zebrafis Pgr, Chen *et al*. 2010). The observed higher EC_{50} value for the cod Pgr might be due to its (partial) inability to interact with co-activators in HEK 293T cells. However, it is also possible that intratesticular DHP levels in cod are relatively high.

In mammals, birds, and amphibians, P4 is considered to be the main ligand for their PGRs. Comparison of the LBD of the cod Pgr with that of PGRs from other species indicated that cod Pgr has more similarity to the teleost forms (81-88%) than to tetrapods PGRs (67-69%), which suggests a greater likelihood of differences in ligand specificity between teleost and tetrapod species.

In cod embryos, there was no maternal contribution of *pgr* mRNA, but from early zygotic expression, the *pgr* mRNA levels steadily increased during larval development, which is in line with previous study in zebrafish (Chen *et al*. 2010). Interestingly, the expression levels of larvae started to show a more important interindividual variation when approaching the size range, in which gonadal sex differentiation can be observed morphologically in Atlantic cod (20-30 mm, unpublished data). Research in zebrafish also revealed that *pgr* mRNA expression was

significantly higher in ovarian than in testicular tissue in early sex-differentiating gonads (Chen *et al*. 2010). These data suggest that Pgr may have a role during early ovarian development in teleost.

The major aim of this study was to reveal possible function(s) of Pgr in mediating DHP-stimulated spermatogenic process of a gadoid species, the Atlantic cod, by quantifying and localizing the testicular expression *pgr* transcripts during one reproductive cycle. In Salmonidae and Cyprinidae high levels of circulating progestins were observed during the spermiation process, and progestins have been proposed to induce spermiation (Ueda *et al*. 1985), to increase seminal fluid production (Baynes and Scott 1985; Yueh and Chang 1997), and to stimulate spermatozoa motility (Miura *et al*. 1992). However, the mechanisms by which progestin exerts these effects are still unclear. Recent results suggest that membrane-associated progestin receptor α (mPR*α*), which is expressed in germ cells, mediates the non-genomic actions of progestins to induce sperm hypermotility in Atlantic croaker and sertrout (Tubbs & Thomas 2008). However, in some teleosts it has been suggested that DHP directly activates sperm carbonic anhydrase, resulting in an increase of pH in seminal plasma, in turn increasinge the cAMP content in sperm, which could be involved in the acquisition of sperm motility (Miura and Miura 2003). In male rainbow trout (*Oncorhynchus mykiss*), DHP treatment induced changes in seminal fluid sodium/potassium concentrations (Baynes and Scott 1985). However, other studies failed to repeat this observation in trout (Milla *et al*. 2008), and DHP was shown to have no effect on potassium transport by the sperm duct of brook trout (*Salvelinus fontinalis*) (Marshall *et al*. 1989). In the present study, although we have no information on circulating or intratesticular progestin levels, the *pgr* mRNA expression level was up-regulated when spermiation started and reached peak levels in spawning testes, which suggests that Pgr may be functionally related to the induction of spermiation, sperm maturation, and perhaps seminal fluid composition in the cod. Unfortunately, detection of *pgr* mRNA by *in situ* hybridization required the use of cryosections, which failed due to technical problems in fully mature testes filled with spermatozoa.

A previous report described high spermatogonial proliferation activity and formation of new spermatogonial cysts in the periphery of the lobes during the period of rapid testis growth (Almeida *et al*. 2008), which suggest that both the cellular composition and RNA yield of the testis changes considerably during early spermatogenesis. Therefore, in order to think over other possible Pgr-mediated effects during earlier stages of testis maturation, RNA yield, testis mass and body weight were introduced to correct the relative gene expression levels. The results indicated that entering the period of rapid testicular growth, characterized by massive spermatogonial proliferation, a first up-regulation of testicular *pgr* mRNA levels was observed, suggesting a role of Pgr in mediating relatively early steps in spermatogenesis. This is supported by the *in situ* hybridization data showing that cod *pgr* mRNA is located predominantly in Sertoli cells in contact with these rapidly proliferating spermatogonia. A similar localization of *pgr* mRNA in testis has been found in zebrafish (Chen *et al*. 2010) and in Atlantic salmon (unpublished data).

In summary, we have cloned a single cDNA coding for a Pgr in the Atlantic cod. Pharmacological characterization indicated its natural ligand is DHP. The expression pattern of *pgr* mRNA during early stage of ontogenesis imply a potential role for sex differentiation towards female. Cellular localization and quantification of *pgr* mRNA in cod testis during onset of spermatogenesis suggest that the cod Pgr, which is expressed in Sertoli cells, may be involved in the regulation of early spermatogenesis and induction of spermiation.

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References

Almeida FFl, Taranger GL, Norberg B, Karlsen O, Bogerd J, Schulz RW 2009 Photoperiod-modulated testis maturation in Atlantic cod (*Gadus morhua*, L.). *Biology of Reproduction* **80** 631-640.

Almeida FFL, Kristoffersen C, Taranger GL, Schulz RW 2008 Spermatogenesis in Atlantic cod (*Gadus morhua*): a novel model of cystic germ cell development. *Biology of Reproduction* **78** 27-34.

Bogerd J, Blomenrohr M, Andersson E, van der Putten HH, Tensen CP, Vischer HF, Granneman JC, Janssen-Dommerholt C, Goos HJ, Schulz RW 2001 Discrepancy between molecular structure and ligand selectivity of a testicular follicle-stimulating hormone receptor of the African catfish (*Clarias gariepinus) Biology of Reproduction* **64** 1633-1643.

Boonyaratanakornkit V, Scott MP, Ribon V, Sherman L, Anderson SM, Maller JL, Miller WT, Edwards DP 2001 Progesterone receptor contains a proline-rich motif that directly interacts with SH3 domains and activates c-Src family of tyrosine kinases. *Molecular Cell* **8** 269-280.

Baynes SM, Scott AP 1985 Seasonal variations in parameters of milt production and in plasma concentration of sex steroids of male rainbow trout (*Salmo gairdneri*). *General and Comparative Endocrinology* **57** 150-160.

Chen SX, Bogerd J, García-López A, de Jonge H, de Waal PP, Hong WS, Schulz RW 2010 Molecular cloning and functional characterization of a zebrafish nuclear progesterone receptor. *Biology of Reproduction* **82** 171-181.

Clarke CL, Sutherland RL 1990 Progestin regulation of cellular proliferation. *Endocrine Revieww* **11** 266-301.

Conneely OM, Mulac-Jericevic B, DeMayo F, Lydon JP, O'Malley BW 2002 Reproductive Functions of Progesterone Receptors. *Recent Progress in Hormone Research* **57** 339-355.

Conneely OM, Maxwell BL, Toft DO, Schrader WT, O'Malley BW 1987 The A and B forms of the chicken progesterone receptor arise by alternate initiation of translation of a unique mRNA. *Biochemical and Biophysical Research*

Communications **149** 493-501.

Evans RM 1988 The steroid and thyroid hormone receptor superfamily. *Science* **240** 889-895.

Hanna RN, Daly SC, Pang Y, Anglade I, Kah O, Thomas P, Zhu Y 2010 Characterization and expression of the nuclear progestin receptor in zebrafish gonads and brain. *Biology of Reproduction* **82** 112-122.

Ikeuchi T, Todo T, Kobayashi T, Nagahama Y 2002 A novel progesterone receptor subtype in the Japanese eel, *Anguilla japonica*. *FEBS Letters* **510** 77-82.

Kastner P, Krust A, Turcotte B Stropp U, Tora L, Gronemeyer H, Chambon P 1990 Two distince estrogen-regulated promoters generate transcripts encoding the two functionally different human progesterone receptor forms A and B. The *EMBO Journal* **9** 1603-1614.

Kusakabe M, Nakamura I, Evans J, Swanson P, Young G 2006 Changes in mRNAs encoding steroidogenic acute regulatory protein, steroidogenic enzymes and receptors for gonadotropins during spermatogenesis in rainbow trout testes. *Journal of Endocrinology* **189** 541-554.

Leal MC, Cardoso ER, Nóbrega RH, Batlouni SR, Bogerd J, França LR, Schulz RW 2009 Histological and stereological evaluation of zebrafish (*Danio rerio*) spermatogenesis with an emphasis on spermatogonial generations. *Biology of Reproduction* **81** 177-187.

Marshall WS, Bryson SE, Idler DR 1989 Gonadotropin stimulation of K+ secretion and Na^+ absorption by brook trout (Salvelinus fontinalis) sperm duct epithelium. *General and Comparative Endocrinology* **75** 118-128.

Milla S, Terrien X, Sturm A, Ibrahim F, Giton F, Fiet J, Prunet P, Le Gac F 2008 Plasma 11-deoxycorticosterone (DOC) and mineralocorticoid receptor testicular expression during rainbow trout *Oncorhynchus mykiss* spermiation: implication with 17*α*, 20*β*-dihydroxyprogesterone on the milt fluidity? *Reproductive Biology and Endocrinology* **6** 19.

Mitteholzer C, Andersson E, Consten D, Hirai T, Nagahama Y, Norberg B 2007 20*β*-hydroxysteroid dehydrogenase and CYP19A1 are differentially expressed during maturation in Atlantic cod (*Gadus morhua*). *Journal of Molecular Endocrinology* **39** 319-328.

Miura C, Ohta T, Ozaki Y, Tanaka H, Mirua T 2009 Trypsin is a multifunctional factor in spermatogenesis. *Proceedings of the National Academy of Sciences of the United States of America* **106** 20972-20977.

Miura T, Higuchi M, Ozaki Y, Ohta T, Miura C 2006 Progestin is an essential factor for the initiation of the meiosis in spermatogenetic cells of the eel. *Proceedings of the National Academy of Sciences of the United States of America* **103** 7333-7338.

Miura T, Miura CI 2003 Molecular control mechanisms of fish spermatogenesis. *Fish Physiology and Biochemistry* **28** 181-186.

Miura T, Kasugai T, Nagahama Y, Yamauchi K 1995 Acquisition of potential for sperm motility in vitro in Japanese eel (*Anguilla japonica*). *Fisheries Science* **61** 533-534.

Miura T, Yamauchi K, Takahashi H, Nagahama Y 1992 The role of hormones in the acquisition of sperm motility in salmonid fish. *Journal of Experimental Zoology* **261** 359-363.

Nagahama Y 1997 17*α*,20*β*-dihydroxy-4-pregnen-3-one, a maturation-inducing hormone in fish oocytes: Mechanisms of synthesis and action. *Steroids* **62** 190-196.

Norberg B, Brown CL, Halldorsson O, Stensland K, Björnsson BT 2004 Photoperiod regulates the timing of sexual maturation, spawning, sex steroid and thyroid hormone profiles in the Atlantic cod (*Gadus morhua*). *Aquaculture* **229** 451–467.

Thornton JW 2001 Evolution of vertebrate steroid receptors from an ancestral estrogen receptor by ligand exploitation and serial genome expansions. *Proceedings of the National Academy of Sciences of the United States of America* **98** 5671-5676.

Todo T, Ikeuchi T, Kobayashi T, Kajiura-Kobayashi H, Suzuki K, Yoshikuni M, Yamauchi K, Nagahama Y 2000 Characterization of a testicular 17*α*,20*β*-dihydroxy-4-pregnen-3-one (a spermiation-inducing steroid in fish) receptor from a teleost, Japanese eel (*Anguilla japonica*). *FEBS Letters* **465** 12-17.

Truss M, Beato M 1993 Steroid hormone receptors: interaction with deoxyribonucleic acid and transcription factors. *Endocrine Reviews* **14** 459-479.

Tubbs C, Thomas P 2008 Functional characteristics of membrane progestin receptor alpha (mPR*α*) subtypes: a review with new data showing mPR*α* expression in seatrout sperm and its association with sperm motility. *Steroids* **73** 935-941.

Ueda H, Kanbegawa A, Nagahama Y 1985 Involvement of gonadotrophin and steroid hormones in spermiation in the amago salmon, *Oncorhynchus rhodurus*, and goldfish, *Carassius auratus*. *General and Comparative Endocrinology* **59** 24-30.

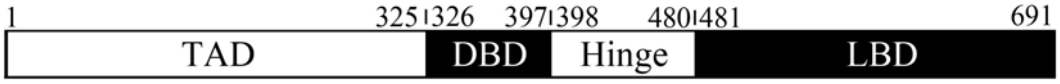
Vogl AW, Vaid KS, Guttman JA 2008 The Sertoli cell cytoeskeleton. Advances in *Experimental Medicine and Biology* **636** 186-211.

Walton MJ, Bayne RA, Wallace I, Baird DT, Anderson RA 2006 Direct effect of progestogen on gene expression in the testis during gonadotropin withdrawal and early suppression of spermatogenesis. *Journal of Clinical Endocrinology & Metabolism* **91** 2526-2533.

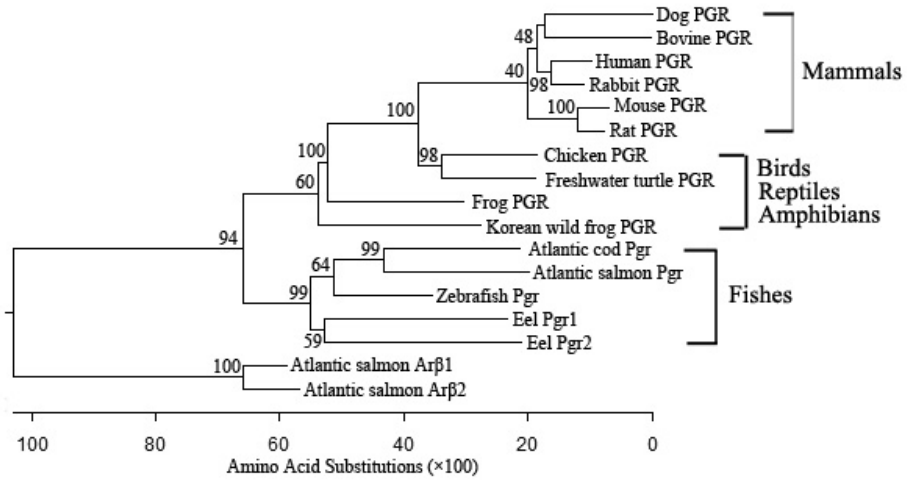
Wittbrodt J, Meyer A, Schartl M 1998 More genes in fish? *Bioessays: News and Reviews in Molecular, Cellular and Developmental Biology* **20** 511-515

Yueh WS, Chang CF 1997 17*α*,20*β*,21-trihydroxy-4-pregnen-3-one and 17*α*,20*β*-dihydroxy-4-pregnen-3-one stimulated spermiation in protandrous black porgy, Acanthopagrus schlegeli. *Fish Physiology and Biochemistry* **17** 187-193.

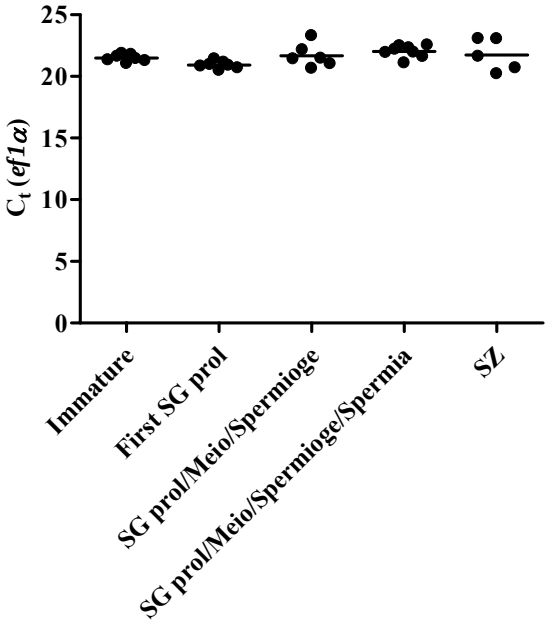
Supplemental information



Supplemental Figure 1 Domains of the cod progesterone receptor.
Schematic representation of the cod Pgr and its domains: TAD, transactivation domain; DBD, DNA-binding domain; Hinge, hinge domain; LBD, ligand-binding domain. Previously, the TAD, DBD, hinge and LBD domains were named A/B, C, D and E/F domains, respectively. The numbers above each box refer to the amino acid positions delimiting the domains.



Supplemental Figure 2 Phylogenetic tree of PGRs.
The phylogenetic tree was constructed by the neighbor-joining method using the MegAlign program (Lasergene software package; DNASTAR Inc.), including only sequences where progesterone binding had been demonstrated experimentally, and rooted by two types of salmon androgen receptor (unpublished data). The horizontal distances to the branching points are proportional to the number of amino acid substitutions. The numbers next to the branches indicate bootstrap values from 1000 replicates.



Supplemental Figure 3 C_t values of *ef1α* mRNA during onset of spermatogenesis. One-way ANOVA was used to compare groups formed on the basis of stage of gonadal development; significant differences were not found. Males were assigned to groups based on the testis histology, with abbreviations indicating: SG Prol, spermatogonia proliferation; Meio, meiosis; Spermioge, spermiogenesis; Spermia, spermiation; SZ, spermatozoa.

Supplementary Table 1

Primer name	Sequence
2197[a]	5'-GAAGAGVAGGARGGCCTTCATGCAGAGRAA-3'
2198[a]	5'-CTCACHTGTGGMAGCTGCAAGGTKTTCTTCAA-3'
2228	5'-GGGGCAAAGTATAAGAATTCACTGGTGACGTTCTTGAA-3'
2229	5'-ACATGCCTATTGCCTTGAGGGCCCCGAAAC-3'
2235	5'-GGCTGGGATGATGCTAGGAGGGAGGAAGTTG-3'
2236	5'-CTGGAGAACATCGAGCCGGAGATGGCGTACT-3'
2259[b]	5'-CTGGCCACCATGGAGAATAAACCCAATGGGAGGAT-3'
2260[c]	5'-TCCCTCCCATTGGTTTATTTCCCACCTCACTTGGT-3'
3068	5'-GAAGGCCATCATTCTGCTCAAC-3'
3069	5'-GCTGGTGGATGGCTTTGGT-3'
3197[d]	5'-[T3Rpp]-CCGGGCATCCGAGAGGTGCAG-3'
3130[e]	5'-[T7Rpp]-TTTATTTCCCACCTCACTTGGTGTGGAACAG-3'

[a]Primers 2197 and 2198 are degenerative primers, in which H = A, T or C, K = G or T, M = A or C, R = G or A, and V = G, A or C.

[b]Primer 2259 contains a Kozak consensus sequence (underlined; Kozak, 1984).

[c]Primer2260 contains 10 nucleotides that are not present in the pgr cDNA sequence.

[d]Primer 3197 contains the T3 RNA polymerase promoter sequence (underlined) at its 5'-end (T3Rpps; 5'-GGGCGGGTGTTATTAACCCTCACTAAAGGG-3').

[e]Primer 3130 contains the T7 RNA polymerase promoter sequence (underlined) at its 5'-end (T7Rpps; 5'-CCGGGGGGTGTAATACGACTCACTATAGGG-3').

Supplemental Table 2 Percentage amino acid identity of the salmon Pgr compared with PGRs from other species.

	Full length	TAD	DBD	Hinge	LBD
Zebrafish Pgr	67	26	97	67	83
Salmon Pgr	67	30	100	58	88
Eel Pgr1	62	21	96	59	81
Eel Pgr2	59	19	90	58	81
Human PGR	33	10	90	38	69
Dog PGR	33	10	90	38	69
Bovine PGR	51	9	90	37	69
Mouse PGR	34	9	90	39	68
Rat PGR	34	8	90	40	69
Rabbit PGR	34	9	90	43	69
Chicken PGR	44	9	90	41	68
Frog PGR	52	8	89	45	67
Korean wild frog PGR	48	9	84	43	67
Freshwater turtle PGR	43	11	90	45	69

The amino acid sequences of the different progesterone receptors were gathered from the GenBank database (see below) and domain by domain homology analysis with salmon Pgr was performed using MegAlign/DNASTAR software. See Figure 1 for a key to the domain nomenclature.

Zebrafish (*Danio rerio*) (FJ409244), Japanese eel (*Anguilla japonica*) (receptor 1, BAA89539; and 2 AB028024), human (Homo sapiens) (M15716), dog (Canis lupus familiaris) (AF177470), bovine (Bos Taurus) (AY656812), mouse (Mus musculus) (M68915), rat (Rattus norvegicus) (L16922), rabbit (Oryctolagus cuniculus) (M14547), chicken (Gallus gallus) (P07812), frog (Xenopus laevis) (AF279335), Korean wild frog (Rana dybowskii) (AF431813), freshwater turtle (Pseudemys nelsoni) (AB301062). Domain by domain homology analysis with the zebrafish Pgr was performed using MegAlign/DNASTAR software. See supplemental figure 1 for key to the specific domains nomenclature.

Chapter 6

六

Summarizing Discussion

It was only 2 years after its discovery in the blood plasma of female sockeye salmon *Oncorhynchus nerka* (Walbaum; Idler *et al.* 1960) that DHP was also identified in the blood plasma of male sockeye and Atlantic salmon (*Salmo salar* L.; Schmidt and Idler, 1962). During the past decades, the main research interest in DHP in male fish addressed the strong association in several species between elevated DHP plasma concentrations and spermiation (Scott and Baynes 1982), and as a potent pheromone in synchronizing spawning behavior (Dulka *et al*. 1987). However, potential roles of DHP during early stages of spermatogenesis have been neglected for a long time, even though a small peak of DHP during the progression of spermatogonial proliferation in salmonid fish has been reported repeatedly (Dépêche and Sire 1982; Scott and Sumpter 1989; Vizziano *et al.* 1996), possibly because of too little "thinking outside the box". After all, plasma androgen levels increased with the start, and further increased with the progression of spermatogenesis (Billard *et al*. 1982), and mainly androgens were considered to stimulate the onset and progression of spermatogenesis (Miura *et al*. 1991). Recently, however, an important role for DHP has been detected during spermatogenesis, namely as initiator of meiosis in Japanese eel (Miura *et al*. 2006).

In context with the remarkable development of research methodologies, new approaches to study the mechanism of action of steroid hormones became available, and many new functions of steroid hormones were uncovered. Therefore, in order to further our understanding of the roles of progestin on fish spermatogenesis, we first cloned and characterized the nuclear progesterone receptor.

Cloning and pharmacological characterization of teleost nuclear progesterone receptor

It has been suggested that the six related steroid receptors (SRs), androgen receptor (AR), estrogen receptor (ER)-1 and -2, progesterone receptor (PGR), glucocorticoid receptor, and mineralcorticoid receptor, arose by a series of gene duplications of an ancestral steroid receptor. In this model, both AR and PGR arose from a gonadal 3-keto steroid receptor by the third genome duplication during vertebrate evolution (Thornton 2001). Moreover, another whole genome duplication 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), so that for many genes, ray-finned fish have two paralogous copies (Wittbrodt *et al*. 1998).

In teleost fish, two distinct paralogous copies of *pgr* genes have been cloned in Japanese eel (*Anguilla japonica*). However, only one *pgr* gene, where the gene product was shown to exhibit progesterone dependent activation, was cloned in zebrafish (Chapter 1, Hanna *et al*. 2010), Atlantic salmon (Chapter 3), and Atlantic cod (Chapter 4). Moreover, extensive *in silico* approaches did not provide evidence for the existence of additional *pgr*-like genes or mRNA isoforms from one gene in zebrafish, Atlantic salmon, medaka, Takefugu, Tetraodon, and stickleback (Chapters 3

and 4; Hanna *et al*. 2010). Thus, it is likely that, in some teleost fish, one *pgr* gene was lost after the teleost-specific genome duplication. A similar situation exists with regard to the Ar, of which two distinct paralogous copies have been identified in a number of fish species, while only one *ar* gene is present in the zebrafish genome (Hossain *et al*. 2008; de Waal *et al*. 2009).

In human, rodents and chicken, two isoforms (A and B), encoded by a single *PGR* gene but originating from different translational initiation sites at two in-phase ATG codons, have been reported (Conneely *et al*. 1987; Kastner *et al*. 1990). Studies using PGR knockout mice indicated that only PGR-A is both necessary and sufficient to elicit the progesterone-dependent reproductive responses required for female fertility, while PGR-B is required for eliciting the normal proliferative responses of the mammary gland to progesterone (Conneely *et al*. 2002). Seen from this evolutionary point of view and considering the predominant gonadal expression of the *pgr* genes cloned in this thesis, we assume that the *pgrs* from zebrafish, Atlantic salmon, and Atlantic cod have important function in the reproductive physiology, the main subject area of this thesis.

The structural features of the cloned *pgr* cDNAs are (i) a variable transactivation domain (TAD) at the N-terminus, (ii) a highly conserved DNA binding domain (DBD), (iii) a hinge region, and (iv) a conserved ligand binding domain (LBD) at the C-terminus (Evans 1988). Although the DBD and LBD are highly conserved, comparison of these two domains in different species indicated that the LBD displayed higher variation during the evolution of vertebrates. When compared with cod Pgr, the percentage identity of the LBDs separated into two groups that are formed by teleost fish (81-88%) and tetrapods (67-69%), respectively. In tetrapod vertebrates (mammals, birds, and amphibians), P4 is considered to be the main ligand for their PGRs. In the present study, we demonstrated that DHP is the major native ligand for the teleost Pgrs. Therefore, identification of unique residues in teleost LBDs, differing from conserved residues found across tetrapod species, could be used as a starting point for analyzing teleost Pgr binding affinity and specifity for DHP in future studies.

Cellular localization of Pgr in testis

In males, the expression of the PGR and the physiological function of progesterone at the molecular level are not well understood (Oettel and Mukhopadhyay 2004). Mature male PGR-knock out mouse have a normal reproductive phenotype, normal serum LH, and mildly decreased FSH levels compared with wild-type animals (Schneider *et al*. 2005), results which suggest that the physiological role of progestins in the male hypothalamic-pituitary axis is minimal, at least in mice. Walton *et al*. (2006) demonstrated that the synthetic progestin desogestrel, when combined with testosterone and a GnRH antagonist, mediated a direct effect on testicular gene expression in humans. Moreover, alterations in the PGR expression pattern in the human testis have been linked to infertility (Abid S *et al*. 2008). In teleost fish, progestins have been identified as essential factors for initiating meiosis in the testis

of Japanese eel (Miura *et al*. 2006). To better illustrate the PGR-mediated progesterone function on the regulation of testicular functions, the cellular localization of the *pgr* transcript in the testis needs to be investigated.

Studies on different mammalian species reported that PGR protein shows localization restricted either to germ cells at different stages of development, or to somatic cells, including peritubular and Leydig cells (Kohler *et al*. 2007; Sirivaidyapong *et al*. 2001; Luetjens *et al*. 2006). In Japanese eel, RT-PCR studies detected *pgr1* mRNA in germ cells, Sertoli cells, and interstitial cells of testis, whereas *pgr2* mRNA was detected only in germ cells (Miura *et al*. 2006). In the present study, using *in situ* hybridization, *pgr* mRNA specific signals were observed in Sertoli cells from all three fish species; in zebrafish, *pgr* mRNA was also detected in Leydig cells. However, no signal was recorded in germ cells of all three species, suggesting that progestin exerts biological activity via the somatic cells in the testis. Interestingly, another group reported Pgr protein expression only in spermatogonia and spermatocytes in zebrafish (Hanna *et al*. 2010). While these different results may be due to different techniques applied (*in situ* hybridization versus immunocytochemistry), it is important to note that in zebrafish, *pgr* mRNA levels did not differ in testis tissue from wild-type animals and from germ cell-depleted mutants (Chapter 1), and that purified germ cells also were negative for *pgr* mRNA. Considering moreover possible mechanisms of action of DHP, in Japanese eel, the DHP-mediated stimulation of spermatogenesis involved elevated expression of the short form of 11*β*-hydroxysteroid dehydrogenase (11*β*Hsd) and of trypsinogen in Leydig and Sertoli cells, respectively (Ozaki *et al*. 2006, Miura *et al*. 2009), suggesting that the Pgr target genes expressed in these somatic cells of the testis may function in regulating spermatogenesis and steroidogenesis.

Roles of Pgr in mediating DHP-stimulated testicular function

As mentioned in the last section, in juvenile Japanese eel, DHP up-regulated 11*β*Hsd expression, the enzyme catalyzing the final step in the production of the main androgen in fish, 11-KT (Ozaki *et al.* 2006). Considering that the Pgr was expressed in zebrafish Leydig cells, we set out to test if DHP-stimulated 11-KT production also in adult zebrafish testis, and if the effect was Pgr-dependent. Our results suggest that this stimulation also occurred in adult zebrafish testis. Furthermore, our study indicated this stimulation was implemented in a Pgr-dependent manner. Taken together, these data indicate that one of roles for DHP would be to modulate the intratesticular levels of other steroid hormones by increasing 11*β*Hsd activity, an enzyme that is required for the production of 11-KT or that catabolises cortisol. However, *pgr* mRNA is expressed in Sertoli cells in both eel and zebrafish, and therefore, we hypothesized that DHP may have additional functions in fish spermatogenesis, independent of a modulation of androgen biosynthesis or cortisol catabolism.

To address this hypothesis, we first studied the effects of exposure to DHP *in vivo*, using a recently developed experimental model based on an oestrogen-mediated

depletion of androgen production to interrupt zebrafish spermatogenesis. Morphological analysis showed, in line with our previous observations (de Waal *et al*. 2009), an effective depletion of differentiating germ cells in testes of zebrafish exposed to E_2 for 5 weeks. Additional exposure to DHP for the final 2 weeks restored spermatogenesis such that all differentiating germ cell stages were abundantly present. Moreover, analysis of a DNA-synthesis marker (BrdU) showed high activity in testis tissue of DHP-treated animals, confirming that DHP treatment stimulated germ cell proliferation and differentiation *in vivo*. Although the expression levels of several genes (*amh, gsdf*, *igf2b*, *igf3,* and *igfr1b*) were significantly higher in testis showing clearly elevated spermatogenetic activity in response to DHP *in vivo*, further studies using an *ex vivo* culture system indicated that only *igfr1b* transcripts were directly up-regulated in testis tissue after DHP treatment. Since unpublished data from our laboratory showed that *igfr1b* mRNA levels did not differ in testis tissue from wild-type animals and from germ cell-depleted mutants, an important component of *igfr1b* expression can be attributed to somatic cells in the testis. Finally, the DHP-induced up-regulation of *igfr1b* mRNA levels in testicular explants exposed to DHP *ex vivo* was similar to after DHP exposure *in vivo*, although the fold up-regulation of germ cell marker genes was more prominent *in vivo* than *ex vivo*. Taken together, the data suggests that the stimulatory effect of DHP on spermatogonial proliferation, differentiation, and entry into meiosis was mediated via Pgr expressed in the Leydig cells and the Sertoli cells, and involves up-regulation of testicular paracrine/autocrine signalling systems, such as the Igf system. However, the stronger effects of DHP *in vivo* compared to *ex vivo* studies suggests that next to direct actions on the testicular level, DHP might target regulatory mechanisms operating in the brain and/or pituitary, potentially leading to an elevated Fsh signalling *in vivo*.

After having raised the possibility that DHP stimulates early spermatogenesis via Pgr, we intended to study if there is a positive relation between *pgr* expression and early spermatogenesis. To this end, we made use of Atlantic salmon as experimental model, since spermatogenesis in this species proceeds largely synchronous, so that many spermatocysts contain germ cells in the same stages of spermatogenesis during the pubertal development. Moreover, this allowed extending our observation to another order of teleost fish.

As mentioned before, salmon *pgr* mRNA expression was restricted to Sertoli cells surrounding spermatogonia. Quantification of *pgr* mRNA in salmon testis in relation to plasma DHP levels and germ cell development suggested that the salmon Pgr may be involved in the regulation of early spermatogenesis, not only with regard to the entry into meiosis, as previously shown for the eel (Miura *et al*. 2006), but also with regard to the differentiation of late type B spermatogonia.

To further confirm the role of Pgr during early spermatogenesis in teleosts, Atlantic cod, a member of the order of Gadiformes, was chosen as experimental animal. In line with studies in zebrafish and Atlantic salmon p*gr*, the cod *pgr* is expressed in Sertoli cells, especially in those in contact with rapidly proliferating spermatogonia. Moreover, the quantification of *pgr* mRNA in cod testis during the onset of spermatogenesis indicated that the cod Pgr, as suggested from studies in zebrafish and

Atlantic salmon, may be involved in the regulation of early or mitotic phase of spermatogenesis. However, the receptor was strongly up-regulated in testis tissue approaching the spawning condition, a stage we could not investigate in the available set of salmon samples, suggesting that a role for the Pgr during the final stages of the reproductive cycle (e.g. spermiation or composition of seminal fluid) may be widespread among teleost fish.

Final remarks

The endocrine regulation of fish spermatogenesis on the testicular level became clear for the fist time using pubertal eel testicular organ culture system, which has demonstrated that the initiation of spermatogonial proliferation toward meiosis is regulated by 11-KT (Miura *et al*. 1991). Later on, research on the same animal model, using same culture system, showed that spermatogonial stem cell renewal is regulated by E_2 (Miura *et al*. 1999), and that the initiation of meiosis is regulated by DHP (Miura *et al*. 2006). However, it is important to note that Igf1 was present in the basal culture medium, which in the presence of sex steroid, plays an essential role as permissive factor in the onset, progress, and regulation of spermatogenesis in the testis of the Japanese eel (Nader *et al*. 1999).

Our research on the adult zebrafish model has demonstrated that the 11-KT supports complete spermatogenesis using an *ex vivo* organ culture system for adult zebrafish testis explants, in which the basal medium is free of growth factors (Leal *et al*. 2009). Moreover, preliminary data indicated that recombinant sea bream Igf1 stimulated zebrafish spermatogenesis in this culture system (Leal *et al*. 2006). Taken together, these results indicate that in zebrafish sex steroids and Igf can independently stimulate spermatogenesis. Ongoing research in our group has demonstrated that 11-KT can induce specifically *igf3* expression *ex vivo*, while other Igf ligands show no response. Moreover, Fsh, which also induces androgen production (García-López *et al*. 2010), has an even stronger stimulatory effect on *igf3* mRNA levels while it is important to state that this effect in not mediated by androgens. In present study, DHP induced *igf1rb* expression both *in vivo* and *ex vivo*, but no other igf ligand or receptor. Taken together, the mitotic phase of spermatogenesis could be supported by a model, in which Fsh activates both growth factor expression as well as steroidogenesis. One of the 11-KT actions could be to contribute to stimulating *igf3* expression, while DHP could support the continuation of spermatogenesis by induction of *igf1rb* expression. These complementary roles of 11-KT and DHP could be supported by a model, in which 11-KT can inhibit the expression of P450c17, facilitating DHP production via an increased availability of substrate for DHP synthesis (Miura *et al*. 2006).

Based on this hypothetical model, it would be interesting to investigate in future studies the cellular location and binding specificity between Igf ligands and their receptors. Moreover, it may be necessary to reconsider the interaction between the Igf signalling system and other growth factors relevant for spermatogenesis, since a merely permissive role, as suggested for the eel, does not seem to "fill the bill" in zebrafish, and possibly in other species (e.g. tilapia). For example, activin B promotes

spermatogonial differentiation and is stimulated by 11-KT in the eel (Miura *et al*. 1995). But is it possible that activin B is a downstream mediator of Igfs? Actually, ongoing research on female zebrafish indicates the recombinant human IGF1 stimulates activin subunit expression (unpublished data). In summary, under the overall support by gonadotropins, a potential steroid-Igf axis may operate in concert with other paracrine factors in the fish testis to regulate spermatogenesis.

References

Abid S, Gokral J, Maitra A, Meherji P, Kadam S, Pires E, Modi D 2008 Altered expression of progesterone receptors in testis of infertile men. *Reproductive BioMedicine Online* **17** 175-184

Billard R, Fostier A, Weil C, Breton B 1982 Endocrine control of spermatogenesis in teleost fish. *Canadian Journal of Fisheries and Aquatic Sciences* **39** 65-79

Conneely OM, Mulac-Jericevic B, DeMayo F, Lydon JP, O'Malley BW 2002 Reproductive functions of progesterone receptors. *Recent Progress in Hormone Research* **57** 339-355.

Conneely OM, Maxwell BL, Toft DO, Schrader WT, O'Malley BW 1987 The A and B forms of the chicken progesterone receptor arise by alternate initiation of translation of a unique mRNA. *Biochemical and Biophysical Research Communications* **149**:493-501.

de Waal PP, Wang DS, Nijenhuis WA, Schulz RW, Bogerd J 2008 Functional characterization and expression analysis of the androgen receptor in zebrafish (*Danio rerio*) testis. *Reproduction* **136** 225-234.

de Waal PP, Leal MC, García-López A, Liarte S, de Jonge H, Hinfray N, Brion F, Schulz RW, Bogerd J 2009 Oestrogen-induced androgen insufficiency results in a reduction of proliferation and differentiation of spermatogonia in the zebrafish testis. *Journal of Endocrinology* **202** 287-297.

Dépêche J, Sire O 1982 In vitro metabolism of progesterone and 17α-hydroxyprogesterone in the testis of the rainbow trout, *Salmo gairdneri* Rich., at different stages of spermatogenesis. *Reproduction, Nutrition, Development* **22** 427-438.

Dulka JG, Stacey NE, Sorensen PW, van der Kraak GJ 1987 A steroid sex pheromone synchronizes male-female spawning readiness in goldfish. *Nature* **325** 251-253.

Evans RM 1988 The steroid and thyroid hormone receptor superfamily. *Science* **240** 889-895.

García-López A, de Jonge H, Nóbrega RH, de Waal PP, van Dijk W, Hemrika W, Taranger GL, Bogerd J, Schulz RW 2010 Studies in zebrafish reveal unusual cellular expression patterns of gonadotropin receptor messenger ribonucleic acids in the testis and unexpected functional differentiation of the gonadotropins. *Endocrinology* **151** 2349-2360.

Hanna RN, Daly SC, Pang Y, Anglade I, Kah O, Thomas P, Zhu Y 2010 Characterization and expression of the nuclear progestin receptor in zebrafish

gonads and brain. *Biology of Reproduction* **82** 112-122.

Hoegg S, Brinkmann H, Taylor JS, Meyer A 2004 Phylogenetic timing of the fish-specific genome duplication correlates with the diversification of teleost fish. *Journal of Molecular Evolution* **59** 190-203.

Hossain MS, Larsson A, Scherbak N, Olsson PE, Orban L 2008 Zebrafish androgen receptor: isolation, molecular, and biochemical characterization. *Biology of Reproduction* **78** 361–369.

Idler DR, Fagerlund UHM, Ronald AP 1960 Isolation of pregnen-4-ene-17*α*,20*β*-diol-3-one from plasma of Pacific salmon (*Oncorhynchus nerka*). *Biochemical and Biophysical Research Communications* **2** 133-137.

Kastner P, Krust A, Turcotte B Stropp U, Tora L, Gronemeyer H, Chambon P 1990 Two distince estrogen-regulated promoters generate transcripts encoding the two functionally different human progesterone receptor forms A and B. *The EMBO Journal* **9** 1603-1614.

Kohler C, Riesenbeck A, Hoffmann B 2007 Age-dependent expression and localization of the progesterone receptor in the boar testis. *Reproduction in Domestic Animals* **42** 1-5.

Leal MC, Skaar KS, de Françac LR, Schulz RW 2006 The effects of IGF-I on zebrafish testis in tissue culture. *Animal Reproduction: Proceedings of the International Symposium on Animal Biology of Reproduction, Nov, 15-18, 2006, Belo Horizonte, MG, Brazil* **3** 181.

Leal MC, de Waal PP, García-López A, Chen SX, Bogerd J, Schulz RW 2009 Zebrafish (*Danio rerio*) primary testis tissue culture: an approach to study testis function *ex vivo*. *General and Comparative Endocrinology* **162** 134-138.

Luetjens CM, Didolkar A, Kliesch S, Paulus W, Jeibmann A, Bocker W, Nieschlag E, Simoni M 2006 Tissue expression of the nuclear progesterone receptor in male non-human primates and men. *Journal of Endocrinology* **189** 529-539.

Miura T, Yamauchi K, Takahashi H, Nagahama Y 1991 Hormonal induction of all stages of spermatogenesis in vitro in the male Japanese eel (*Anguilla japonica*). *Proceedings of the National Academy of Sciences of the United States of America* **88** 5774-5778.

Miura T, Miura C, Yamauchi K, Nagahama Y 1995 Human recombinant activin induces proliferation of spermatogonia in vitro in the Japanese eel **(***Anguilla japonica*). Fisheries Sciences **61** 434-437.

Miura T, Miura C, Ohta T, Nader MR, Todo T, Yamauchi K 1999 Estradiol-17*β* stimulates the renewal of spermatogonial stem cells in males. *Biochemical and Biophysical Research Communications* **144** 5504-5510.

Miura T, Higuchi M, Ozaki Y, Ohta T, Miura C 2006 Progestin is an essential factor for the initiation of the meiosis in spermatogenetic cells of the eel. *Proceedings of the National Academy of Sciences of the United States of America* **103** 7333-7338.

Miura C, Ohta T, Ozaki Y, Tanaka H, Mirua T 2009 Trypsin is a multifunctional factor in spermatogenesis. *Proceedings of the National Academy of Sciences of*

the United States of America **106** 20972-20977.

Nader MR, Miura T, Ando N, Miura C, Yamauchi K 1999 Recombinant human insulin-like growth factor I stimulates all stages of 11-ketotestosterone-induced spermatogenesis in Japanese eel, *Anguilla japonica*, in vitro. *Biology of Reproduction* **61** 944-947.

Oettel M, Mukhopadhyay AK 2004 Progesterone: the forgotten hormone in men? *Aging Male* 7 236-257.

Ozaki Y, Higuchi M, Miura C, Yamaguchi S, Tozawa Y, Miura T 2006 Roles of 11*β*-hydroxysteroid deheydrogenase in fish spermatogenesis. *Endocrinology* **147** 5139-5146.

Schmidt PJ, Idler DR 1962 Steroid hormones in the plasma of salmon at various stages of maturation. *General and Comparative Endocrinology* **2** 201-214.

Schneider JS, Burgess C, Sleiter NC, DonCarlos LL, Lydon JP, O'Malley B, Levine JE 2005 Enhanced sexual behaviors and androgen receptor immunoreactivity in the male progesterone receptor knockout mouse. *Endocrinology* **146** 4340-4348.

Scott AP, Baynes SM 1982 Plasma levels of sex steroids in relation to ovulation and spermiation in rainbow trout (*Salmo gairdneri*). In *Reproductive Physiology of Fish* (Richter CJJ & Goos HJT eds), pp. 224-234. St John's: Marine Science Research Laboratory.

Scott AP, Sumpter JP 1989 Seasonal variations in testicular germ cell stages and in plasma concentrations of sex steroids in male rainbow trout (*Salmo gairdneri*) maturing at 2 years old. *General and Comparative Endocrinology* **73** 46-58.

Sirivaidyapong S, Bevers MM, Gadella BM, Colenbrander B 2001 Induction of the acrosome reaction in dog sperm cells is dependent on epididymal maturation: the generation of a functional progesterone receptor is involved. *Molecular Reproduction and Development* **58** 451-459.

Thornton JW 2001 Evolution of vertebrate steroid receptors from an ancestral estrogen receptor by ligand exploitation and serial genome expansions. *Proceedings of the National Academy of Sciences of the United States of America* **98** 5671-5676.

Vizziano D, LeGac F, Fostier A 1996 Effect of 17*β*-estradiol, testosterone, and 11-ketotestosterone on 17*α*,20*β*-dihydroxy-4-pregnen-3-one production in the rainbow trout testis. *General and Comparative Endocrinology* **104** 179-188.

Walton MJ, Bayne RA, Wallace I, Baird DT, Anderson RA 2006 Direct effect of progestogen on gene expression in the testis during gonadotropin withdrawal and early suppression of spermatogenesis. *The Journal of Clinical Endocrinology and Metabolism* **91** 2526-2533.

Wittbrodt J, Meyer A, Schartl M 1998 More genes in fish? *Bioessays: News and Reviews in Molecular, Cellular and Developmental Biology* **20** 511-515.

SUMMARY

The present thesis expanded the knowledge on the roles of progestins and its nuclear receptor (Pgr) in fish spermatogenesis. Using zebrafish, our results indicated that effect of progestin on 11betaHsd activity was via Pgr expressed in Leydig cells. Quantification of *pgr* mRNA in Atlantic salmon testis in relation to other reproductive parameters suggested that the Pgr expressed in the Sertoli cells may be involved in the differentiation of late type B spermatogonia and the entry into meiosis. In line with studies in Atlantic salmon, studies in Atlantic cod indicated that the Pgr may be involved in the regulation of the late mitotic and meiotic phases of spermatogenesis. Moreover, the receptor was strongly up-regulated in cod testis tissue approaching the spawning, suggesting roles for the Pgr during the final stages of the reproductive cycle. Subsequently, using an *in vivo* experimental model, and an *ex vivo* testis culture system, we could show that DHP treatment induced the proliferation of early spermatogonia as well as their differentiation into late spermatogonia and spermatocytes in zebrafish. Further, transcripts of Sertoli cell-derived growth factor genes were up-regulated, but only *igf1rb* mRNA levels showed a significant increase under DHP treatment *in vivo* and *ex vivo*.

SAMENVATTING

Dit proefschrift heeft de kennis verruimd m.b.t. de rol van progesteron and zijn nucleaire receptor (Pgr) voor de zaadcelvorming bij vissen. Gebruikmakend van de zebravis als experimenteel model werd aangetoond dat één van de effecten van progesteron, nl. de verhoging van de 11β-hydroxysteroïd dehydrogenase activiteit, gemedieerd wordt door de Pgr die in Leydigcellen tot expressie komt. Kwantificering van een aantal parameters relevant voor de voortplanting en van de hoeveelheid *pgr* mRNA in de Sertolicellen van de zalm suggereerde dat deze receptor een rol zou kunnen spelen bij de differentiatie van late type B spermatogoniën en de start van de meiose. In overeenstemming met de resultaten verkregen bij de zalm wezen studies bij de kabeljauw erop dat de Pgr ook hier een rol speelt bij de laatste mitotische delingsstappen van de spermatogoniën en de meiotische fase van de zaadcelvorming. Bovendien werd een sterke verhoging van de testiculaire Pgr expressie gemeten bij benadering van de paaitijd zodat de Pgr ook tijdens de laatste stadia van de voortplantingscyclus een rol zou kunnen spelen. Vervolgens kon m.b.v. *in vivo* en *ex vivo* weefselkweek experimenten aangetoond worden, weer onder gebruik van de zebravis als model, dat een progesteron behandeling niet alleen de proliferatie van vroege spermatogoniën induceerde, maar ook hun differentiatie naar late spermatogoniën en spermatocyten. Bovendien bleek de expressie van een aantal groeifactor genen in Sertolicellen verhoogd te zijn, maar het was alleen de hoeveelheid igf1 receptor type B mRNA dat in de aanwezigheid van progesteron zowel *in vivo* als ook *ex vivo* verhoogd was.

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Curriculum Vitae

I was born on the 28th of November 1980 in Fuzhou (means "lucky area" in english), a city in the southeast of China. I has completed my Marine Biology studies with a Bachelor degree in Xiamen University in July 2003. From September 2003, I continued my education by starting a Master student project at the State Key Laboratory of Marine Environmental Science & Department of Oceanography, Xiamen University, China. In September 2005, according to the regulations of Xiamen University, I was selected as a PhD candidate, and privileged to not have to write and defend his MA thesis for the MA diploma.

In 2007, I had applied for and succeeded in obtaining a grant from the "China Scholarship Council", enabling me to spend two years in The Netherlands to complete his PhD thesis work. The intention of this stay is to get acquainted with and to apply, cell and molecular biological techniques to questions related to the biological function of progestin and its nuclear receptor in the reproductive physiology of male fish.

List of Publications

Chen S.X., Bogerd J., García-López A., de Jonge H., de Waal P.P., Hong W.S. & Schulz R.W. (2010) Molecular cloning and functional characterization of a zebrafish nuclear progesterone receptor. *Biology of Reproduction* **82**:171-181.

Chen W., **Chen S.X.** & Hong W.S. (2009) Cardiac performance of Chinese black sleeper (Bostrichthys sinensis) at early stages of development under hypoxic conditions. *Journal of the Marine Biological Association of the United Kingdom* 89(7):1409-1413.

Wu R.X., Hong W.S., Zhang Q.Y. & **Chen S.X.** (2009) Comparative enzyme activities of the intestinal brush border membranes of the herbivorous mudskipper Boleophthalmus pectinirostris and the carnivorous Chinese black sleeper *Bostrischthys sinensis*. *Journal of applied ichthyology* 25:571-575.

Leal M.C., de Waal P.P., García-López A., **Chen S.X.**, Bogerd J. Schulz R.W. (2009) Zebrafish primary testis tissue culture: an approach to study testis function ex vivo. *General and Comparative Endocrinology* 162(2):134-138.

Chen S.X., Hong W.S., Su Y.Q. & Zhang Q.Y. (2008) Microhabitat selection in the early juvenile mudskipper *Boleophthalmus pectinirostris*. *Journal of Fish Biology* 72:585-593.

Wang Q., Hong W.S., **Chen S.X.** & Zhang Q.Y. (2008) Variation with semilunar periodicity of plasma steroid hormone production in the mudskipper *Boleophthalmus pectinirostris*. *General and Comparative Endocrinology* 155:821-826.

Chen S.X., Hong W.S., Zhang Q.Y. & Su Y.Q. (2007) Why does the mudskipper *Boleophthalmus pectinirostris* build territories in farming pond? *Journal of the Marine Biological Association of the United Kingdom* 87:615–619.

Hong W.S., **Chen S.X.**, Zhang Q.Y. & Wang Q. (2007) Reproductive ecology of mudskipper, *Boleophthalmus pectinirostris*. *Acta Oceanologica Sinica* 26(4):72-81.

Hong W.S., **Chen S.X.**, Zhang Q.Y. & Zheng W.Y. (2006) Sex organ extracts and artificial hormonal compounds as sex pheromones to attract broodfish and to induce spawning of Chinese black sleeper (*Bostrichthys sinensis* Lacépède). *Aquaculture Research* 37:529-534.

Hong W.S., **Chen S.X.**, Xiao Y., Zhang Q.Y. & Zheng W.Y. (2006) Hermaphroditism in Cultured Chinese Black Sleeper *Bostrichthys sinensis* L. *Journal of the World Aquaculture Society* 37(4): 363-369.

Chen S.X., Hong W.S., Zhou W. & Zhang Q.Y. (2006) Hypoxic tolerance of Chinese black sleeper *Bostrichthys sinensis* embryos at heartbeat stage. *Journal of the Marine Biological Association of the United Kingdom* 86:1473-1476.

Chen S.X., Hong W.S., Zhang Q.Y., Wu R.X. & Wang Q. (2006) Rates of oxygen consumption and tolerance of hypoxia and desiccation in Chinese black sleeper (*Bostrichthys sinensis)* and mudskipper (*Boleophthalmus pectinirostris*) embryos. *Acta Ocenologica Sinica* 25(4):91-98.