Hormonal regulation of spermatogenesis in zebrafish

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Hormonale regulatie van spermatogenese in zebravis
(met een samenvatting in het Nederlands)

Proefschrift

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Table of Contents

1

General Introduction

Experimental models in male reproductive endocrinology: from eunuchs to zebrafish

Figure 1. Arnold Adolph Berthold (1803-1861); founding father of classical endocrinology

The first scientific evidence on the existence of the endocrine system did not emerge before the 19th century, but observations on a relation between the gonads and secondary sexual characteristics are of all times. In the great Egyptian and Chinese dynasties (before 500 BC) observations were made on reduced male characteristics, such as aggression, in castrated servant eunuchs, and, Aristotle (384-322 BC), as the first experimenter, has described the effects of castration in several species. Groundbreaking and starting endocrinology research was the work by Berthold (1803-1861) (Fig 1). He observed that the testis, when transplanted in the abdominal cavity of castrated roosters, prevented the post-gonadectomy decline of male behaviour and regression of male secondary sexual characteristics (*e.g.* comb size). The detection of newly generated vasculature in the transplanted testis contributed to the hypothesis that substances produced in the testis enter the blood and sustain male traits (Berthold 1849). Alcohol extracts from large quantities of bovine testicles could remasculinize castrated roosters, rats and pigs (Moore and Gallagher 1929). From this extract the substance responsible for remasculinization was purified (David 1935) and its structure was solved (Butenandt and Hanisch 1935). The substance was called testosterone (testo=testicle, ster=sterol, one=ketone; T).

A governing role of the pituitary on gonadal function and T secretion was proposed by a number of studies describing gonadal atrophy or persistence of sexual immaturity after ablation of the anterior part of the organ (Crowe *et al*. 1910; Smith 1926, 1927, 1930; Zondek 1926). The 'gonadotrophic principle' that the pituitary governs gonadal function and T secretion by the release of hormones was suggested by the induction of precocious sexual maturation after replacement of anterior pituitary glands of several species into immature female and male rodents (Smith 1926; Zondek 1926). The discovery of two pituitary-secreted hormones, "Follicle Ripening Hormone" (*i.e* follicle-stimulating hormone; FSH) and "Luteinizing Factor" (*i.e.* luteinizing hormone; LH) (Zondek 1930), and their

specific actions (Fevold *et al*. 1931), was the basis for the concept of control of FSH on spermatogenic processes and of LH over androgen production in the testis (Greep *et al*. 1936; Greep and Fevold 1937), still accepted to the present day. Since then endocrinologists have investigated the distinct actions of the gonadotropins and androgens on male reproduction in a wide variety of vertebrates finding subtle differences in mechanisms and sites of action between species (Steinberger 1971). Over the years, rodents (*e.g*. rats and mice) were studied most frequently, by far, mainly because of their high numbers of offspring, and since recent times because of the opportunities of genetic engineering. However, for applied purposes, cattle and other domesticated species have been studied as well. Non-human primates are equally well-studied since they represent a model most closely mimicking the human condition.

Fish, more specifically, teleosts (*i.e.* bony fish), represent the most numerous group of species among vertebrates inhabiting fresh and marine waters worldwide. Adaptations to the diverse environments led to the evolution of many reproductive strategies. One teleost species of considerable interest is the zebrafish (*Danio rerio*), a small (4-7 cm in length; 0.4-0.8 g in body weight) tropical fresh water fish, which belongs to the family of *Cyprinidae* and the order of *Cypriniformes*, to which goldfish (*Carassius auratus*) and common carp (*Cyprinus carpio*) belong as well. Zebrafish originate from Indian waters near the Himalaya region, where they spawn once a year; however, domesticated laboratory zebrafish strains reproduce throughout the year (Spence *et al*. 2008). The ongoing Sanger Zebrafish Genome project (www.sanger.ac.uk/Projects/D_rerio) has added tremendously to the popularity of zebrafish as an easy-to-handle and robust laboratory model for which detailed protocols exist, in order to generate and maintain laboratory stocks (Westerfield 2000). For example, gene and protein nomenclatures for fish follow the standards of the Official Zebrafish Nomenclature Guidelines (http://zfin.org; *gene/mrna* and Protein in fish, whereas *Gene/Mrna* and PROTEIN in *e.g*., mice). Furthermore, application of reverse genetic strategies by ENU mutagenesis, in order to elucidate gene function in mapped phenotypes, has raised considerable interest (Wienholds *et al.* 2003). In addition, the zebrafish has emerged as an attractive laboratory model to study reproductive endocrinology, but has been used also to study effects of endocrine disrupting chemicals, for example (McGonnell and Fowkes 2006).

Piscine spermatogenesis

Sexual differentiation in zebrafish is characterized by juvenile hermaphroditism: all individuals develop ovarian structures in week 2-3 post-hatching; later on, around week 4 after hatching, when male gonadal sex differentiation starts, the gonad, undergoes an ovary-to-testis transformation, during which ovarian structures degenerate and testicular structures become apparent (Takahashi 1977; Uchida *et al.* 2002; Wang *et al.* 2007). Zebrafish reach sexual maturity depending on the strain around 90 days post fertilization (dpf). A hallmark of male sexual maturation is the activation of germ cell production, or spermatogenesis.

Spermatogenesis is a cyclic, complex and tightly orchestrated process, involving cellular, paracrine and endocrine interactions between multiple cell types and organs. General characteristics of spermatogenesis are shared by all vertebrate species. Germ cells undergo a transformation process, in which single, diploid spermatogonial stem cells, of which a subpopulation has the capacity to develop in many different cell lineages under the appropriate conditions (Guan *et al.* 2006; Gallicano *et al.* 2009), develop into small, highly specialized, haploid cells, the spermatozoa. Generally accepted is that spermatogonial stem cells in vertebrates 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 are committed to differentiate further in a multi-step process into spermatozoa. The balance between the two types of divisions is coordinated tightly; when the balance is shifted toward stem cell self-renewal seminomas can arise, and when shifted toward differentiation the stem cell population can become depleted and spermatogenesis stops (de Rooij and Grootegoed 1998). Recently the mode of spermatogonial stem cell division has been debated (Oatley and Brinster 2008), and the co-existence of an intermediary type of division was suggested, where a spermatogonial stem cell divides asymmetrically to provide for a stem cell and a daughter cell committed to differentiation (Luo *et al.* 2009). The differentiation pathway of germ cells into spermatozoa can be divided roughly in three phases: (i) the spermatogonia go through a mitotic phase, during which a series of first slow and then rapid divisions expand the germ cell number. Germ cells then enter (ii) the meiotic phase, during which their paternal and maternal DNA is recombined and segregated during two meiotic divisions, the last without DNA synthesis, resulting in haploid set of chromosomes. Finally, during (iii) the spermiogenic phase the haploid germ cells transform into flagellated spermatozoa.

The entire process takes place in the intratubular compartment of the testis, which contains the germinal epithelium of the testis. The only other intratubular

cell type, next to germ cells in different stages of development, is the Sertoli cell. The Sertoli cell has been characterized for the first time in 1865 by Enrico Sertoli (1842-1910), who related this "branched" cell type to the production of spermatozoa (Hess and De França 2005). Sertoli cells are interconnected with tight junction complexes, and contribute to the blood-testis-barrier, separating the interstitial from the intratubular compartment. Furthermore, they provide physical, nutrient and paracrine support in their close and intimate contact with germ cells by establishing a microenvironment suitable for either stem cell renewal (*i.e.* the spermatogonial stem cell niche [De Rooij 2009]) or the differentiation of germ cells.

It is in the association of Sertoli cells and germ cells, where spermatogenesis in anamniotes (amphibian and fish) differs from spermatogenesis in amniotes (mammals, birds and reptiles). Amphibian and piscine spermatogenesis occurs in so-called spermatogenic cysts. The formation of a cyst occurs when one or more Sertoli cells envelope an undifferentiated A type spermatogonium, possibly the spermatogonial stem cell in these species (Pudney 1995). Sertoli cells nurse the subsequent differentiation of a single group of germ cells, which are clonal descendents of this stem cell in a synchronized stage of development (Fig 2A). The expansion of germ cells within the cyst is accompanied by Sertoli cell proliferation, also in the adult piscine testis, occurring mainly during the spermatogonial phase and levelling off during meiosis (Schulz *et al.* 2005; Leal *et al.* 2009). In contrast, spermatogenesis in amniotes (mammals, reptilians, birds) occurs in the seminiferous tubules, where one Sertoli cell at a given time can support germ cells, each in a different stage of development and potentially descending from different stem cells,. Moreover, these Sertoli cells cease to proliferate in the testis after their pubertal maturation.

The spermatogonial or mitotic phase of spermatogenesis provides for exponential germ cell expansion and is subject to regulation (de Rooij and Russell 2000). Therefore, it is an essential phase in establishing the reproductive capacity in a species. A fixed number of divisions occur, which can vary among species. In piscine spermatogenesis, uniform classification of the different spermatogonial generations was missing until recently, when a respective attempt was made (Schulz *et al.* 2009; Leal *et al.* 2009). The following classification manner applies for zebrafish and is based on nuclear size, shape, distribution of heterochromatin, presence of nucleoli and number of spermatogonia per cyst (Fig. 2B; Leal *et al.* 2009). The undifferentiated, single spermatogonia can be divided in A_{und*} and A_{und}. Spermatogonia differentiate and undergo 3 mitotic divisions into type A-differentiated (A_{diff}) spermatogonia ($A_{diff}1$, $A_{diff}2$ and $A_{diff}3$). In five subsequent

mitotic divisions the, now, B type spermatogonia expand substantially in numbers. The later spermatogonial generations as well as the transition of meiotic cells into the spermiogenic phase are determined as periods sensitive to germ cell loss by apoptosis (Leal *et al.* 2009). Apoptosis is a normal, regulated feature of spermatogenesis.

The last generation of B-type spermatogonia enters the meiotic phase. Events during the meiotic phase are similar among vertebrate species. The first meiotic division consists of a lengthy prophase during which pairing of homologous chromosomes occurs. The homologous chromosomes are held into place by the synaptonemal complex (containing the commonly used primary spermatocyte marker, synaptonemal complex protein 3) for proper meiotic recombination as they pass leptotene, zygotene, pachytene stages. The complex disintegrates in diplotene stages before the newly recombined chromosomes are segregated. Subsequently, primary spermatocytes complete their first meiotic division, giving rise to secondary spermatocytes, which complete their second meiotic division immediately without DNA synthesis. The formed haploid spermatids undergo maturational cellular transformation processes into motile, flagellated spermatozoa, (*i.e.* spermiogenesis) and are released into the lumen by the opening of the cyst (*i.e.* spermation).

Next to the intratubular compartment, the other compartment of the testis is the interstitial compartment, which holds blood vessels, nerve fibres, macrophages, mast cells, peritubular myoid cells, fibroblasts and Leydig cells. Leydig cells are the sex steroid-producing cells in the testis, where cholesterol undergoes enzymatic modifications in a process commonly referred to as steroidogenesis (Fig. 2A); the main product of the Leydig cells are the male sex steroids or androgens.

Endocrine control of spermatogenesis by the hypothalamic-pituitary-testis axis: presenting the players

The processes of spermatogenesis and steroidogenesis are under control of the glycoprotein hormones, FSH and LH, and the sex steroids, androgens, produced in Leydig cells. FSH and LH are secreted by pituitary gonadotroph cells under control of gonadotropin-releasing hormone (GnRH), which is released by the hypothalamus. In mammals, FSH and LH have distinct functions, different sites of action, and specifically bind to their respective cognate membrane-associated G-protein-coupled receptors (GPCRs), the FSH receptor (FSHR) and the LH receptor (LHR), which are expressed in the testis by Sertoli and Leydig cells, respectively

(Pierce and Parsons 1981; Saez 1994; Kumar 2005; Petersen and Soder 2006). Leydig cell steroid hormone synthesis is primarily controlled by LH, whereas Sertoli cell activities, such as supplying germ cells with physical, nutritional and paracrine support, are controlled by FSH (Saez 1994; Petersen and Soder 2006). In contrast, promiscuity in ligand specificity, especially of the Fshr for Lh in high concentrations (Bogerd *et al.* 2001; So *et al.* 2005; García-López *et al.* 2009), and overlapping testicular expression sites of the piscine gonadotropin receptors (Ohta *et al.* 2007; García-López *et al.* 2009), suggest less distinctive roles of Fsh and Lh in fish testicular physiology. Indicative of this, is the potent steroidogenic potential of piscine Fsh proteins displayed in multiple species (Planas and Swanson 1995; Kazeto, et al. 2008; García-López *et al.* 2009, in preparation).

Under gonadotrophic control, Leydig cells are stimulated to produce androgens mediated mainly by the cAMP/protein kinase A second messenger cascade (Stocco *et al.* 2005; García-López *et al.* 2009). In all vertebrates quick transport of cholesterol to the inner membrane of mitochondria is provided by steroidogenic acute regulatory protein (StAR). Cholesterol is converted there by the cytochrome P450 side-chain cleavage (CYP11A1) enzyme to pregnenolone, the rate-limiting step of steroidogenesis (Arukwe 2008). Pregnenolone leaving the mitochondria is the substrate for all other steroids including androgens. Since fish Leydig cells show high cytochrome P450 11β-hydroxylase (Cyp11b) and 11β-hydroxysteroid dehydrogenase (Hsd11b) activities (Kusakabe *et al.* 2006; Wang and Orban 2007), the 11-oxygenated androgen, 11-ketotestosterone (11-KT), features as a physiologically prominent androgen beside testosterone (T) (Borg 1994; Schmidt and Idler 1962).

Androgens exert biological actvity via a member of the nuclear receptor superfamily, the androgen receptor (AR), which acts after dimerisation as a steroid-hormone-activated transcription factor by recruiting the transcription complex, and co-activators and/or co-repressors on androgen response element-containing promoter sequences, thereby regulating the expression of androgen-responsive genes. In mammalian testis, the AR is expressed in Sertoli, Leydig and peritubular myoid cells (Ruizeveld de Winter *et al.* 1991; Bremner *et al.* 1994). Some studies reported AR expression in germ cells, which was not required to achieve normal fertility (Johnston *et al.* 2001; Tsai *et al.* 2006). In a number of fish species, two distinct AR types exist (Takeo and Yamashita 1999; Ikeuchi *et al.* 1999, 2001; Katsu *et al.* 2007), possibly reflecting genome duplication events in piscine evolution (Douard *et al.* 2008). The cellular localization of piscine Ars in testis is still largely unexplored; however, somatic expression was indicated (Ikeuchi *et al.* 2001).

Regulation of spermatogenesis: for adults only

Androgens and FSH are generally regarded as the pivotal hormones regulating spermatogenesis across vertebrates (Plant and Marshall 2001; McLachlan *et al.* 2002; Schulz and Miura 2002; Abe 2004; Allan and Handelsman 2005; Sharpe 2005; Schulz *et al.* 2009). Since Sertoli cells, and not germ cells (as described above), express functional receptors for both FSH and androgens, and since Sertoli cells contact germ cells closely and intimately, it is generally assumed that these "nurse" cells function as regulatory interface between the endocrine system and the microenvironment, in which the germ cells develop (Griswold 1995; Petersen and Soder 2006).

Androgens are crucial for the induction of puberty and the maintenance of spermatogenesis across vertebrates (Miura *et al*. 1991; Nagahama 1994; McLachlan *et al*. 2002; Schulz and Miura 2002; De Gendt *et al*. 2004). In fish, rising 11-KT plasma levels mark the start of spermatogonial proliferation in the annual cycle (Campbell *et al*. 2003). Further increasing 11-KT concentrations were associated with the advancement of spermatogenesis towards spermation (Fostier, *et al*. 1982; Campbell *et al*. 2003). Moreover, 11-KT was shown to induce spermatogenesis by stimulating the differentiation of spermatogonia towards meiosis *in vivo* (Cavaco *et al*. 1998) and *in vitro* (Miura *et al.* 1991; Amer *et al*. 2001). Evidence exists that Sertoli cells mediate 11-KT action on germ cells by providing a microenvironment suitable for differentiation. This can be accomplished by releasing differentiation promoting factors (*e.g.* activin B; Miura *et al*. 1995) or preventing the release of differentiation inhibiting factors (*e.g.* anti-Müllerian hormone, Amh; Miura *et al*. 2002). Androgens, mainly T, exert negative feedback as well, either directly on intratesticular androgen synthesis (Cavaco *et al*. 1999; Schulz *et al*. 2008), or indirectly via its conversion into 17β-oestradiol (E_2) in the pituitary, resulting in reduced gonadotropin synthesis and release (Dickey and Swanson 1998). In rodents, androgen signalling in Sertoli cells is essential for germ cells to progress through meiosis, and Sertoli cell-specific ablation of the *Ar* gene leads to infertility in mice (De Gendt *et al*. 2004). Spermatogonial differentiation, on the other hand, develops normally in these knockout mice (De Gendt *et al*. 2004). Understanding of androgen action on spermatogonial differentiation in rodents is complicated by the observation that recovery of spermatogenesis in immature or germ cell-depleted models (*e.g.* juvenile spermatogonial depletion or hypogonadal mutants, or irradiated mice) needs suppression of intratesticular T concentrations in order to allow spermatogonia to differentiate (reviewed by Meistrich and Shetty 2003).

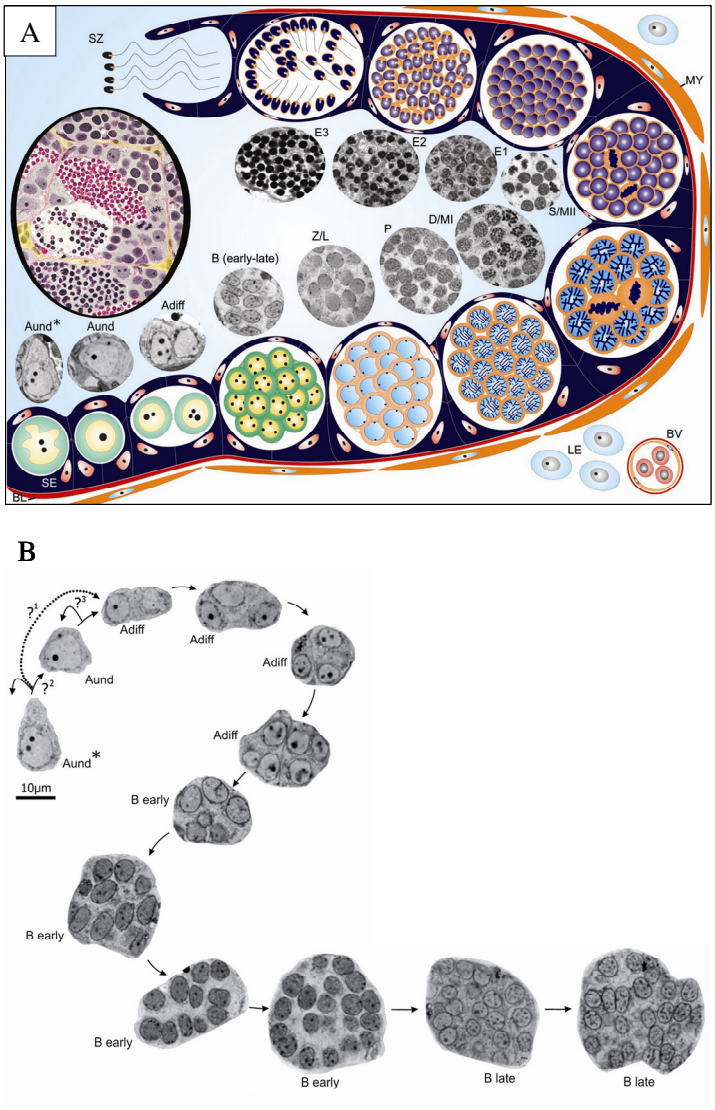

Figure 2. Schematic representation and microphotographs (A) of cystic spermatogenesis and (B) the spermatogonial generations in zebrafish. The germinal epithelium contains Sertoli (**SE**) and germ cells, delineated by a basal lamina (**BL**) and peritubular myoid cells (**MY**). The interstitial Leydig cells (**LE**) and blood vessels (**BV**) are shown. Within the testicular tubules, cysts can be recognized each containing clonal lines of germ cells: Type A undifferentiated* spermatogonia (**Aund***) (stem cell?); type A undifferentiated spermatogonia (**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**); spermatozoa (**SZ**). This figure is adapted from Schulz *et al.* 2009.

In light of the rapid and strong steroidogenic potency of Fsh proteins in fish and the expression of *fshr* mRNA and Fshr protein in Leydig cells (as described above), Fsh action on germ cell development independent of steroids is not well-studied. In eel, Fsh-mediated stimulation of spermatogonial differentiation is abolished when androgen production is blocked (Ohta *et al*. 2007), and rising Fsh plasma levels are accompanied by rising androgen plasma levels at the start of the proliferative spermatogonial phase in male Chinook salmon (*Oncorhynchus tshawytscha*) (Campbell *et al*. 2003). Thus, investigating directly Sertoli cell-mediated Fsh action on testis physiology in fish and its influence on spermatogonial differentiation is highly warranted. However, androgen-mediated Fsh action may contribute highly to testis function *in vivo*. This is in contrast with mammals, where FSHR is expressed solely by Sertoli cells (Themmen *et al*. 1994). Although FSH can not act directly on Leydig cells in mammals, FSH can induce release of paracrine factors by Sertoli cells influencing Leydig cell steroidogenesis (Verhoeven and Cailleau 1986; Grootenhuis *et al*. 1990). FSH is a major controller of mammalian Sertoli cell function (Allan and Handelsman 2005; Petersen and Soder 2006). FSH is important as stimulator of immature Sertoli cell proliferation, in this way regulating the testicular capacity to support germ cells (Baker and O'Shaughnessy 2001). Furthermore, to nurse germ cell proliferation and maturation, Sertoli cells under control of FSH (similar to androgens) establish an appropriate microenvironment via growth factor release (Yamamoto *et al*. 2001; Tadokoro *et al*. 2002; Lukas-Croisier *et al*. 2003; Oral *et al*. 2008). In the amphibian Japanese newt several studies demonstrated that Fsh facilitates all stages of spermatogenesis or at least passage through meiosis in prepubertal Japanese newt (*Cynops pyrrhogaster)* testis (Abe and Ji 1994; Abe 2004; Tanaka *et al*. 2004; Li *et al*. 2008) without induction of androgen production (Tanaka *et al*. 2004).

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. Although hypophysectomy experiments on adult fish did not reveal distinct Fsh or androgen action on spermatogenesis, at least these experiments revealed the spermatogenic phases sensitive to endocrine regulation. Hypophysectomy on adult killfish (*Fundulus heteroclitus*), guppy (*Poecilia reticulata*) and dogfish (*Squalus acanthias*) revealed spermatogenesis was prominently affected during the proliferation of type B spermatogonia, and certain meiotic and spermiogenic events were found affected as well (Billard 1969; Pickford *et al*. 1972; Dobson and Dodd 1977). Additionally, in prolonged hypophysectomised state the differentiation of type A spermatogonia was reduced (Dobson and Dodd 1977).

Distinct aspects of the regulation on spermatogenesis by androgens or FSH were investigated in higher vertebrates. However, many of these studies were conducted before puberty or in models with congenital defects becoming apparent often before puberty, and thereby "possibly confounding proper analysis of the spermatogenic progress after puberty" (McLachlan *et al*. 2002). Mechanisms and sites of action of androgens and FSH in the maintenance of spermatogenesis during adulthood still remain largely elusive across vertebrate species. In order to isolate and investigate the distinct actions androgens or FSH exert on adult spermatogenesis models of gonadotropin withdrawal have been generated; rodents and primates were treated long term with low doses of exogenous T (in some cases supplemented with E_2), with GnRH antagonists or by immunisation against GnRH to deplete LH (and subsequently intratesticular T levels) and/or FSH; acute gonadotropin withdrawal was accomplished with hypophysectomy (reviewed by McLachlan *et al*. 2002). Spermatogenesis was found to be disturbed, and could be attributed to the withdrawal of gonadotropins. Subsequently, attempts were made to restore spermatogenesis with either T or FSH in order to elucidate their distinct mode of action. However, T and FSH action varied considerably among treatments and species (between rodents, primate and human, primarily) (McLachlan *et al*. 2002). For example, what could be distilled from these models is the FSH action on the differentiation from A_{pale} to B type spermatogonia in primates (Marshall *et al*. 2005; O'Donnell *et al*. 2001). Another example is the prevention of apoptosis of spermatogonia and spermatocytes by T or FSH treatment in hypophysectomised rats (El Shennawy *et al*. 1998). However, generally accepted is a great part of complementary action and synergism that androgens and FSH display in the regulation of every phase of adult mammalian spermatogenesis (McLachlan *et al*. 2002; Allan and Handelsman 2005). FSH support is more evident in earlier stages of spermatogenesis (*i.e.* spermatogonial phase and meiosis), whereas the emphasis of androgen support lies more on meiotic and postmeiotic germ cell stages in mammalian species (McLachlan *et al*. 2002; Allan and Handelsman 2005). Moreover in general, short-lived mammals with large numbers of offspring show relatively small effects on the mitotic phase, in contrast to long-lived mammalian species with lower numbers of offspring (McLachlan *et al*. 2002).

Aims and outline of the thesis

In this thesis, efforts were made to understand the endocrine regulation of spermatogenesis, and attention was focussed on, particularly, the spermatogonial

phase. As an experimental model, zebrafish were chosen based on the reproductive capacity in males throughout the year, indicating spermatogenesis is ongoing in adulthood and has to be maintained on a mature level. As mentioned earlier, FSH and androgens act in concert during certain mammalian spermatogenic phases. In salmonid fish, pituitary synthesis and plasma levels of Fsh, and not Lh, rise at the start of spermatogenesis (Gomez *et al*. 1999; Campbell *et al*. 2003), and were accompanied by elevating 11-KT plasma levels (Campbell *et al*. 2003). Piscine Fsh action in prepubertal fish testis was found to be mediated by androgens (Ohta *et al*. 2007). In the present thesis, we focussed on elucidating mechanisms of androgen regulation on adult spermatogenesis but also steroid-independent mechanisms of Fsh action were of considerable interest.

Since information on the pathway of androgen synthesis and on the vital androgen signalling partner, the Ar, in zebrafish testis was missing, the studies described in **Chapter 2** were set out to determine the physiologically relevant androgens and to test their binding and activation potential on the cloned zebrafish androgen receptor (Ar/*ar*). Moreover, *ar* (cellular) expression patterns in zebrafish testis and in a variety of tissues in both sexes were examined.

In order to investigate androgen action on testis physiology isolated from possible feedback effects that androgens provide as well on other components of the hypothalamic-pituitary-testis axis, testicular organ culture systems are a powerful experimental means. Testicular organ culture systems developed for other anamniote species were based mainly on pre-pubertal individuals until now (Miura *et al*. 1991; Abe and Ji 1994). The development of a primary zebrafish testicular organ culture system (described in **Chapter 3)** would allow studies on androgens, gonadotropins and other substances of critical relevance to adult piscine spermatogenesis.

Information on androgen regulation of mammalian spermatogenesis has been gathered from gonadotropin and androgen withdrawal models. Since oestrogens are known to inhibit androgen synthesis, the oestrogen-induced androgen insufficiency zebrafish model described in **Chapter 4** was developed in order to identify the androgen-dependent stages during adult piscine spermatogenesis *in vivo*. Moreover, the mechanisms how oestrogens affect androgen synthesis *in vivo*, were investigated in this chapter.

Androgen action on germ cells is mediated by Sertoli cell-secreted factors, which then promote differentiation of spermatogonia. However, many of these factors are still unknown. Utilizing the models established in the previous sections, **Chapter 5** investigates the insulin-like growth factor (IGF) family as regards possible effects on spermatogenesis. Investigations included expression analysis of

IGF peptides and receptors, a detailed study of the regulation exerted by sex steroids and gonadotropins on mRNA expression of the IGF peptides, and the evaluation of biological activity of a recombinant fish IGF on spermatogenesis.

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

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2

Functional characterization and expression analysis of the androgen receptor in zebrafish (*Danio rerio*) testis

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Abstract

The biological activity of androgens, important for male sexual differentiation and development, is mediated by the androgen receptor that binds to specific DNA recognition sites regulating the transcription of androgen target genes. We investigated androgen production by adult zebrafish testis tissue, and identified 11β-hydroxyandrostenedione, 11-ketoandrostenedione and 11-ketotestosterone as main products, and hence potential ligands, for the zebrafish androgen receptor (Ar). These androgens were then included in the pharmacological characterization of the zebrafish Ar. The zebrafish Ar responded well in terms of binding and transactivation to synthetic androgens as well as to testosterone and 11-ketotestosterone, and reasonably well to 11-ketoandrostenedione and androstenedione. *In situ* hybridization analysis of zebrafish testis revealed that *ar* mRNA expression was detected in the subpopulation of Sertoli cells contacting early spermatogonia.

Introduction

The androgen receptor (AR) is a member of the nuclear receptor family of proteins that function as ligand-activated transcription factors. The AR mediates the masculinising effects of androgens on different parts of the reproductive system at different stages of ontogenesis. For example, androgenic sex steroids are involved in male differentiation of the efferent duct system for germ cells (Hannema & Hughes 2007), spermatogenesis (De Gendt *et al*. 2004), reproductive behaviour and secondary sexual characteristics (Sato *et al.* 2004; Soma 2006). The AR shows a widespread expression pattern over different tissues, suggesting a broad spectrum of androgen-induced biological activities. Also in females, androgens are important for reproductive function, as indicated by the folliculogenesis phenotype in female *Ar* knockout mice (Shiina *et al.* 2006).

Teleost fish are no exception to this general vertebrate pattern, as exemplified by the effects of androgens on secondary sexual characteristics and behaviour (Pall *et al.* 2002a; Pall *et al.* 2002b), on spermatogenesis (Miura *et al.* 1991; Cavaco *et al.* 2001), or on Leydig cell androgen production (Cavaco *et al.* 1999). As regards sex differentiation, fish appear to be particularly sensitive to androgen action, considering that fully functional, female-to-male sex reversal can be induced by exposure of juvenile (Baroiller & Guiguen 2001) and even adult fish (Kobayashi *et al.* 1991) to androgens; in some species, sex change is part of the normal life cycle (Baroiller & Guiguen 2001).

To further our work on zebrafish male sex differentiation and on the development to functional maturity and adult regulation of the two main testicular functions, spermatogenesis and steroidogenesis, and to be able to proceed to studies on the identity and regulation of expression of AR target genes relevant for these processes, we cloned the full-length zebrafish androgen receptor (*ar*) cDNA and studied *ar* mRNA expression by real-time, quantitative PCR and *in situ* hybridization. Moreover, we wanted to identify the physiological ligand(s) for the zebrafish Ar in males. In this context, it is important to note that teleost fish express 11β-hydroxylase (Cyp11b) (Wang & Orban 2007) and 11β-hydroxysteroid dehydrogenase (Hsd11b) (Kusakabe *et al.* 2006) activity in the testis, so that 11-ketotestosterone (11-KT) is a prominent circulating androgen next to testosterone (T) in many species (Schmidt & Idler 1962; Borg 1994). Although respective data are not available in zebrafish, a close relative, the common carp (*Cyprinus carpio*), showed the typical teleost pattern with 11-KT levels being twice as high as the T levels in the plasma of mature males (Koldras *et al.* 1990). We have, therefore, analyzed the main androgens produced by zebrafish testis tissue, which were then included in the pharmacological characterization of the zebrafish Ar. Cloning and quantitative expression analysis of zebrafish *ar* have been published also by others (Jørgensen *et al.* 2007; Hossain *et al.* 2008) very recently, but these studies did not include a detailed comparison between the ligand-binding characteristics and transactivation properties of the zebrafish Ar. Such a comparison is, however, reported in the present study.

Material & Methods

The material and methods section describing the cloning of the full-length zebrafish *ar* cDNA, the phylogenetic analysis of the zebrafish Ar as well as the real-time, quantitative PCR analysis of zebrafish *ar* tissue distribution and ontogeny are presented as Supplemental information, which can be viewed online at www.reproduction-online.org/supplemental/.

Animals and source of steroid hormones

Zebrafish (*Danio rerio*; Tübingen AB strain) were kept at a light-dark cycle of 12h-12h under standard conditions (Westerfield 2000). Animal culture and handling was consistent with the Dutch national regulations; the Life Science Faculties Committee for Animal Care and Use approved the experimental protocols.

All non-radioactive steroids and the AR antagonist flutamide were purchased from Sigma-Aldrich (St. Louis, MO, USA). Steroids used in this study were testosterone (T), 11-ketotestosterone (11-KT), 17α-methyltestosterone (MT), 17α-dimethyl-19-nortestosterone (mibolerone [MB]), androstenedione (A2), 11-ketoandrostenedione (OA), 11β-hydroxyandrostenedione (OHA), 11β-hydroxytestosterone (OHT), 5α-dihydrotestosterone (DHT), 17β-estradiol (E_2), progesterone (P), 17α-hydroxyprogesterone (OHP), 17α-hydroxy, 20β-dihydroprogesterone (OHH_2P) and cortisol.

Zebrafish androgen receptor expression vector construct, cell lines and transfections

The full-length open-reading frame of the zebrafish *ar* was PCR amplified using primers 1943 and 1944 (Suppl. Table 1), cloned into pcDNA3.1/V5-His TOPO vector (Invitrogen) and the insert was sequence verified by DNA sequence analysis.

Since zebrafish is a small species (body weight of an adult male ~ 0.5 gram), it is not feasible to perform ligand-binding studies on target tissue homogenates. Therefore, a human embryonic kidney cell line (HEK 293T cells; DuBridge *et al.* 1987) was used to express the zebrafish *ar*. HEK 293T cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% v/v fetal bovine serum (FBS), non-essential amino acids, glutamine and penicillin/streptomycin (all from Gibco; Carlsbad, CA, USA) at 37°C in a CO_2-incubator. Cells were transfected using a standard calcium phosphate precipitation method (Graham & Van der Eb 1973).

Binding assay

For saturation ligand binding analysis, HEK 293T cells were seeded in 10 cm dishes (~1 x 10^6 cells per dish) and after 24 h co-transfected with 0.1, 1, or 6 µg of zebrafish *ar* expression vector construct and up to 11 µg of carrier plasmid. One day after transfection, the cells were transferred to 24-well plates, coated with poly-L-lysin hydrobromide (Sigma-Aldrich). Two days after the transfer, the cells were incubated for 2 h with binding assay medium (DMEM without phenol red, supplemented with glutamine, non-essential amino acids and charcoal-stripped 0.2% v/v FBS [to remove any steroids originating from the FBS]) at 37°C. Then, radioactive tracer ([^{3}H]-testosterone, [^{3}H]-T; specific activity 77.0 Ci/mmol; PerkinElmer, Waltham, MA, USA) was added, either alone or in the presence of 1 µM unlabeled T, dissolved in binding medium. After 90 min incubation at room temperature, the cells were quickly washed two times with ice-cold phosphate-

buffered saline (PBS) to remove unbound tracer. Cells were harvested in 200 µl of sodium hydroxide (1 M) per well and radioactivity was counted in a β-counter (Packard 1900 TR liquid scintillation counter; Packard Instruments, Meriden, CT, USA). Specific [^{3}H]-T binding over a range of increasing concentrations was calculated by subtracting non-specific binding (binding of tracer in the presence of unlabeled T) from total binding (binding of tracer in the absence of unlabeled T); in all cases, bound [^{3}H]-T could be displaced by increasing concentrations of unlabeled T (data not shown). Pilot experiments with HEK 293T cells, transfected with various amounts of zebrafish *ar* expression vector construct, revealed that the zebrafish Ar displayed nanomolar affinity for [^{3}H]-T (data not shown); in all other experiments, including the ligand competition assays (see below), HEK 293T cells were transfected with 1 µg zebrafish *ar* expression vector construct and 10 µg carrier plasmid. Binding remained unchanged over a period up to 8 h, indicating that [^{3}H]-T is not metabolized in HEK 293T cells (data not shown). Moreover, both non-transfected and mock transfected HEK 293T cells did not show any specific binding of [^{3}H]-T (data not shown). Non-linear curve fitting procedures (GraphPad PRISM 4.0; GraphPad Software Inc.; San Diego, CA, USA) were used to calculate the dissociation constant (K_d).

Ligand competition assay

To determine the affinity of other steroids for the zebrafish Ar, HEK 293T cells transfected with the zebrafish *ar* expression vector construct were incubated with increasing concentrations (10 pM to 1 µM) of each steroid, mixed with tracer ([^{3}H]-T; final concentration 2.7 nM) at room temperature, followed by measurement of tracer binding to the transfected cells. The IC_{50} values were calculated with non-linear regression (GraphPad PRISM 4.0). To allow calculation of K_i values from IC_{50} values, a dose-response curve of non-labelled testosterone was included in each experiment. Assuming that ARs possess the same affinity for T and [^{3}H]-T, K_i values were calculated by using the formula: $K_i = (IC_{50}\,\text{steroid}/IC_{50}\,\text{T}) \times K_d$ [^{3}H]-T.

Transactivation assay

HEK 293T cells were seeded in 10 cm dishes (~1.25 x 10^6 cells per dish). After 24 h, the cells were co-transfected with 500 ng of zebrafish *ar* expression plasmid and 10 µg of MMTV-Luc plasmid (Stocklin *et al.* 1996). One day later, the cells were transferred to 24-well plates coated with poly-L-lysin hydrobromide (Sigma-Aldrich). The next day, the medium was replaced with transactivation assay medium (DMEM without phenol red, supplemented with charcoal-stripped 0.2% v/v FBS, glutamine and non-essential amino acids) containing steroid at end

concentrations ranging between 1 pM and 1 µM. After 24-36 h of incubation at 37°C, cells were harvested in lysis mix (100 mM potassium phosphate [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 [pH 7.7], 250 mM D-luciferin [Invitrogen], 1 mM DTT, 2 mM adenosine triphosphate [Roche, Almere, The Netherlands] and 15 mM magnesium sulphate [Promega, Madison, WI, USA]) to thawed samples and luminescence was measured in a PerkinElmer luminometer.

Analysis of androgen production in zebrafish testis

Except for a study on the production of steroid glucuronides and their possible role as pheromones (Van den Hurk *et al*. 1987), no information has been published on the identity of the main androgenic steroids produced by adult zebrafish testis tissue. To address this caveat, the following experiment was performed in triplicate: testis tissue was collected from 8 adult males (28.7 ± 3.6 mg total wet weight). Each testis was divided into two fragments, and the tissue fragments were pooled, rinsed with L15 medium, and transferred into 2 ml L15 medium containing tritiated A2 (7-[^{3}H]-A2; specific activity 24.5 Ci/mmol; NET1001, NEN Dupont, Boston, MA, USA) at a final concentration of 100 nM. After 15, 30, and 60 min of incubation at 28°C in a gently shaking waterbath (5 revolutions per min), 0.25 ml of medium was removed, added to a tube containing a mixture of 5 µl of each of the following, non-radioactive carrier steroids (20 µg/ml ethanol): T, A2, OHA, OHT, OA, and 11-KT. Steroids were immediately extracted twice with 0.5 ml of dichloromethane. The two aliquots of dichloromethane were combined, evaporated and the extracts were transferred, dissolved in a few drops of ethanol, to thin-layer chromatography plates (10 x 10 cm HPTLC silica-coated glass plates with a 10 x 2.5 cm concentrating zone; Merck, Darmstadt, Germany). The plates were first developed in toluene:cyclohexane = 1:1 to concentrate the samples, and steroids were then separated by developing the plate with chloroform:ethanol = 95:5. The non-radioactive carrier steroids, added just before extraction, were localized under UV light at 254 nm. The plate was then treated with a scintillation spray (En^{3}hance Spray, NEN Dupont), and radioactivity was localized as photons using Hyperfilm™ MP (Amersham Life Science, Piscataway, NJ, USA). To relate bands on the film to the amount of radioactivity associated with the different fractions, the bands were quantified densitometrically using a PC-based image analysis system, using a program developed in the KS400 version 3.0 software package (Carl Zeiss Vision, Göttingen, Germany). Results are expressed as percent

of the total amount of radioactivity of the respective sample. Steroids were identified by co-migration with non-radioactive carrier steroids that were visualized under UV light.

In situ hybridization

A zebrafish *ar*-specific PCR product was generated with primers 2430 and 2431 (see Suppl. Table 1). The ~465 bp PCR product was gel purified, and served as a template for digoxigenin-labelled cRNA probe synthesis, as described previously (Vischer *et al.* 2003).

Zebrafish testes were dissected and fixed in 4% w/v paraformaldehyde in PBS, immersed in 25% w/v sucrose at 4°C for 16 h, and then frozen in Neg-50 frozen section medium (Richard Allen Scientific, Kalamazoo, MI, USA). The protocol used for *in situ* hybridization was described previously (Weltzien *et al.*, 2003) with the following modifications. Cryostat sections were cut at 10 µm thickness, and probe was added in a final concentration of 800 ng/ml. After staining sections were rinsed in 96 % ethanol for 40 s and in MilliQ water for 15 min, before mounting in Aquamount (Merck).

Results

Zebrafish androgen receptor cDNA cloning, phylogenetic and expression analysis

The results section describing the full-length zebrafish *ar* cDNA cloning, the phylogenetic analysis of the zebrafish Ar and the tissue distribution and ontogeny of zebrafish *ar* mRNA expression are presented as Supplemental information, including Supplemental Figures 1-3, which can be viewed online at www.reproduction-online.org/supplemental/.

Androgen production by zebrafish testis tissue

To study the steroid specificity and transactivation capacity of the zebrafish Ar in a targeted manner, we first investigated to which products adult zebrafish testis tissue fragments metabolized 100 nM [^{3}H]-A2 during 15, 30, or 60 min of incubation. Separation of the products by thin-layer chromatography showed that the substrate remained largely unconverted in the absence of tissue (Fig. 1A), but a minor impurity of the substrate (Unk-2) was found. In all cases where [^{3}H]-A2 was incubated with tissue, at least 96% of the radioactivity co-migrated with known carrier steroids. Densitometry of the autoradiogram showed that the substrate was progressively metabolized with time (Fig. 1B). A major metabolite was OHA that

appeared quickly and was prominently present (11-22%) at all time points. The pattern of appearance of OA differed from that of OHA by showing a steady increase with time from 7 to nearly 27% of the total product. This pattern was similar to the one of 11-KT except that the latter accumulated at a lower rate (1.5-11%). Minor products (< 2% at all time points) were T and OHT, and 1-2% of the radioactive products that did not co-migrate with the non-radioactive carrier steroids were assigned to Unk-1 and -3, respectively, while up to 1% was represented by Unk-2, a minor impurity of the substrate also present in the control incubation without tissue. Taken together, these data suggest that A2 is quickly and effectively metabolized to OHA by Cyp11b activity, before being converted to OA by Hsd11b activity. OA is further converted to 11-KT by 17β-hydroxysteroid dehydrogenase (Hsd17b) activity. It appears, however, that neither OHA nor A2 are readily accepted as substrate by the testicular Hsd17b activity, considering that only trace amounts of T or OHT have been found. These results are summarized schematically in Fig. 2.

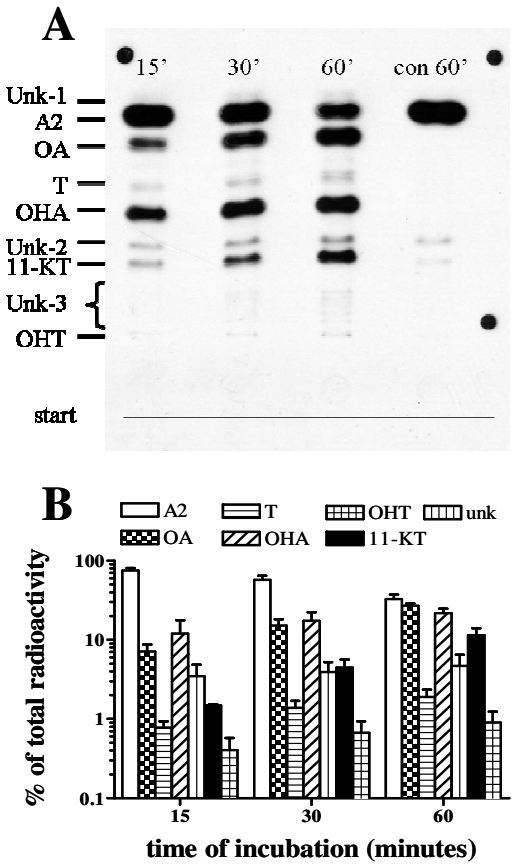

Figure 1 Androgen production in zebrafish testis tissue. A. Representative autoradiogram of a thin-layer chromatogram of dichloromethane-extracted L15 medium containing [^{3}H]-A2 in the presence of zebrafish testis fragments for 15, 30 or 60 min (indicated with 15', 30' and 60') or in the absence of zebrafish testis fragments for 60 min (indicated with con 60'), after which non-radioactive carrier steroids (androstenedione [A2], 11-ketoandrostenedione [OA], testosterone [T], 11β-hydroxyandrostenedione [OHA], 11-ketotestosterone [11-KT], 11β-hydroxytestosterone [OHT]) were added. Unk-1, unk-2 and unk-3 indicate unknown products. B. Quantification of densitometrically scanned bands of the autoradiogram (shown in Fig. 1A.) representing the steroid metabolites of [^{3}H]-A2 conversion in zebrafish testis tissue; each column represents the average radioactivity (± SEM) for a given metabolite, expressed as percentage of the total amount of radioactivity per lane, determined in three independent experiments.

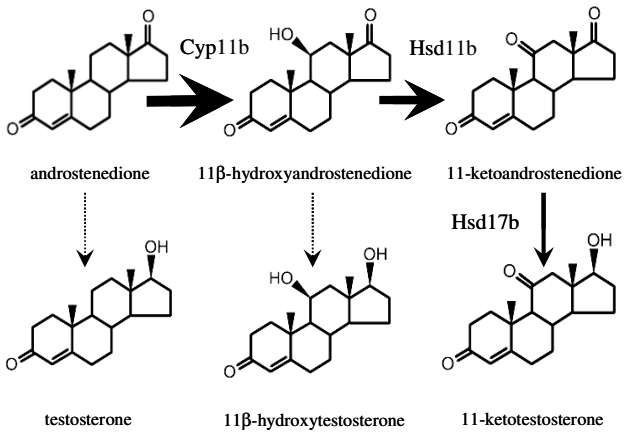

Figure 2 Schematic representation of steroidogenic processing of androstenedione in zebrafish testis (based on the data shown in Fig. 1). The main products of zebrafish testis are 11β-hydroxyandrostenedione (OHA), 11-ketoandrostenedione (OA) and 11-ketotestosterone (11-KT). The main steroidogenic pathway is indicated by thick arrows. Steroidogenic enzymes indicated are: 11β-hydroxylase (Cyp11b), 11β-hydroxysteroid dehydrogenase (Hsd11b) and 17β-hydroxysteroid dehydrogenase (Hsd17b).

Ligand-binding characteristics of the zebrafish androgen receptor

To determine the ligand-binding characteristics of the zebrafish Ar, we first performed saturation ligand-binding assays on HEK 293T cells, transfected with the zebrafish *ar* expression vector construct, using [^{3}H]-T as a tracer. High-affinity and saturable binding of [^{3}H]-T was observed (Fig. 3A), while no specific binding was observed using non-transfected or mock-transfected cells (data not shown). Our analysis clearly indicated that the zebrafish Ar behaved as a single class of high affinity [^{3}H]-T binding protein with a K_d of 1.7 nM.

Zebrafish androgen receptor steroid specificity

To determine the relative affinity of the zebrafish Ar for the most prominent androgens produced in zebrafish testis in comparison with other (synthetic) androgens and non-androgenic steroids, competitive binding assays were performed, using 2.7 nM [^{3}H]-T as a tracer (Table 1 and Fig. 3B). The curves were parallel, indicating competitive binding between the androgens and [^{3}H]-T, allowing IC_{50} values to be determined and K_i values to be calculated. The zebrafish Ar showed the highest affinity for the two synthetic androgens MT and MB (Table 1). Natural androgens, like T and 11-KT, showed nanomolar affinities for the receptor, with T and DHT showing slightly higher affinities for the receptor than 11-KT. Of the androgenic steroids that are biochemical precursors of 11-KT, A2

had a higher affinity for the zebrafish Ar than OA or OHA. Of the non-androgenic steroids, progesterone and its hydroxylation derivatives (OHP and OHH_2P) showed affinities for the receptor in the high nanomolar range, while E_2 and cortisol showed clearly lower affinities to the zebrafish Ar.

Transactivation of the zebrafish androgen receptor

To determine the steroid-induced transactivation properties of the zebrafish Ar, we co-transfected zebrafish *ar* expression vector construct together with an androgen-regulated reporter vector construct (containing the luciferase gene under the control of the MMTV promoter) into HEK 293T cells (see below). When HEK 293T cells were transfected with the MMTV-Luc vector only, androgens did not increase luciferase activity, indicating that HEK 293T cells do not express endogenous ARs (data not shown).

Dose-dependent, zebrafish Ar-mediated activation of the MMTV promoter was shown for several androgens, *e.g.* MT, DHT (an important androgen in higher vertebrates, but not produced in fish), 11-KT and T (Table 1 and Fig. 3C). The synthetic androgen MT was the most potent steroid for the zebrafish Ar (EC_{50} = 0.03 ± 0.01 nM), reaching maximal activation at 1 nM (not shown). The main circulating androgens in fish, 11-KT and T, were somewhat less potent in activating the zebrafish Ar, T being more potent than 11-KT. A2 and OA showed medium to high nanomolar EC_{50} as well as K_i values. OHA, albeit showing a certain binding to the zebrafish Ar, was a weak androgen in terms of Ar-mediated transactivation of the MMTV promoter. The EC_{50} values for all steroids tested are shown in Table 1.

To determine the relative potency of various non-androgenic steroids to transactivate the MMTV promoter via the zebrafish Ar, they were tested at a fixed concentration of 100 nM (Fig. 3D). The androgens 11-KT and T (positive controls) induced clear responses, increasing luciferase activity by 32- and 23-fold, respectively. Of the non-androgenic steroids, only P and OHH_2P were able to induce small but statistically not significant increases in luciferase activity, whereas E_2, cortisol and OHP were inactive. Hence, the surprisingly low K_i concentrations found for some of the non-androgenic steroids (*e.g.* P and OHH_2P) were not associated with the capacity to activate the zebrafish Ar. We can conclude that low K_i concentrations only coincide with low EC_{50} concentrations for activation as well as with effective induction of reporter gene expression in the case of androgens. Among these C19 steroids, the 17β-hydroxylated configuration was most effective while the status of C-atom 11 (with or without an oxygen function, *viz.* T and 11-KT) seemed less relevant. However, when a keto group was

present at C atom 17, the status of C-atom 11 did matter, since androgens with either no oxygen (A2) or a keto group (OA) showed an intermediate affinity and transactivational capacity, while an 11β-hydroxy group (OHA) further reduced binding affinity and abolished biological activity.

Transactivation of the MMTV promoter via the 11-KT-stimulated zebrafish Ar was inhibited by an AR antagonist. The antagonistic effect of flutamide on the zebrafish Ar-mediated MMTV-promoter transactivation via increasing doses of 11-KT (1 pM to 10 µM) was clearly demonstrated (Fig. 3E), since a four-fold or sixty-fold higher concentration of 11-KT was needed to reach 50% of the maximal activation with 11-KT in the presence of 1 µM (EC_{50} = 4.3 nM) or 10 µM (EC_{50}= 64 nM) flutamide, respectively, compared with the condition where no flutamide was included (EC_{50} = 1.2 nM).

Table 1: Comparison of ligand competition data and transactivation data of the zebrafish Ar. K_i values (nM) of several steroids for the zebrafish androgen receptor are given. For reference, the K_d value of radiolabeled testosterone ([^{3}H]-T) is also included. The zebrafish Ar was expressed in HEK293T cells and [^{3}H]-T was used as tracer. K_i values represent average ± SEM of three independent experiments. EC_{50} values of ligand-induced Ar transactivation of the MMTV promoter are also shown. Luciferase activity was measured after incubation with increasing concentrations of various steroid hormones (1 pM to 10 µM). EC_{50} values represent average ± SEM of three independent experiments.

Competitive binding		**Transactivation**		
Steroid	**K_i (nM)**	**Steroid**	**EC_{50} (nM)**	**Max. fold induction**
MT	0.73 ± 0.20	MT	0.03 ± 0.01	40.81 ± 3.93
MB	0.82 ± 0.01	DHT	0.13 ± 0.04	39.69 ± 2.22
DHT	1.65 ± 1.55	T	0.42 ± 0.20	39.33 ± 4.64
[^{3}H]-T (K_d value)	1.70 ± 0.50	11-KT	1.16 ± 0.60	43.70 ± 2.52
OHH_2P	4.64 ± 2.80	MB	1.63 ± 2.79	34.86 ± 3.79
11-KT	4.77 ± 2.26	OA	50.8 ± 34.7	36.38 ± 5.01
P	6.99 ± 1.61	A2	58.0 ± 31.1	16.84 ± 4.17
A2	19.6 ± 1.84	OHH_2P	116 ± 103	8.44 ± 2.19
OHP	21.8 ± 1.34	P	467 ± 615	4.50 ± 1.61
E_2	71.6 ± 96.7	OHP	951 ± 1543	4.50 ± 1.46
OA	71.7 ± 5.24	cortisol	1855 ± 702	10.07 ± 2.55
OHA	128 ± 24.0	OHA	N.D.	2.16 ± 0.26
Cortisol	352 ± 162	E_2	N.D.	1.87 ± 0.26

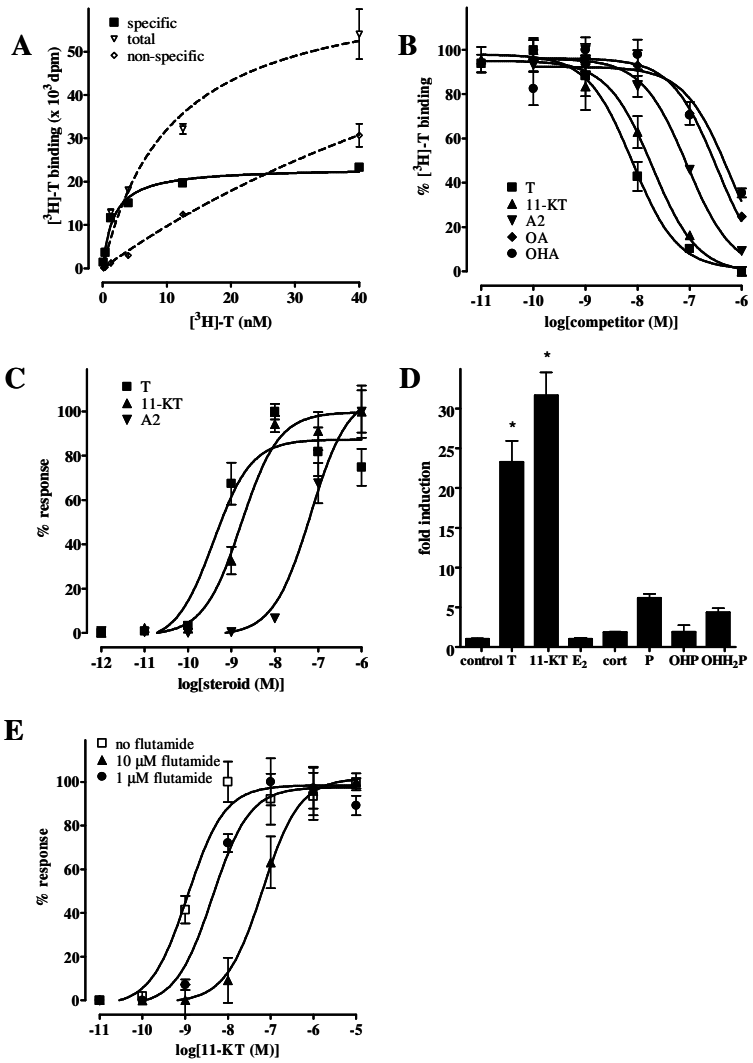

Figure 3 Binding and transactivation analysis of the zebrafish Ar. A. Saturation analysis of zebrafish Ar. HEK 293T cells were transfected with the zebrafish *ar* expression vector construct and incubated with increasing amounts of [^{3}H]-T in the presence or absence of a 1000-fold excess of unlabeled T. Total, non-specific and specific [^{3}H]-T binding are shown. B. Competitive binding analysis of the zebrafish Ar. HEK 293T cells were transiently transfected with the zebrafish *ar* expression vector construct, and incubated with [^{3}H]-T as a tracer in the absence (not shown) or presence of increasing concentrations (10 pM to 1 µM) of various androgens. C. Ligand-induced transactivation properties of the zebrafish Ar. HEK 293T cells were transiently co-transfected with the MMTV-luciferase vector together with the zebrafish *ar* expression vector construct. Cells were incubated with increasing concentrations of various androgens (1 pM to 10µM). D. Ligand-induced, zebrafish Ar-mediated transactivation of the MMTV promoter at a fixed concentration of 100 nM of various steroids. As a control the ratio of the fold induction of OHA at 100 nM (no appreciable induction observed) divided by the fold induction of OHA at 1 pM (no active ligand) is given. In addition, OHA has a very low affinity for the zebrafish Ar. Each column represents the mean ratio of luciferase activity at 100 nM of the steroid divided by the luciferase activity at 1 pM of OHA of three independent experiments, with the vertical bars representing the SEM. Lack of error bars is due to the errors being too small to show graphically. Asterisks represent fold induction significantly different from control, $P<0.001$, using 1-way ANOVA with Newmann-Keuls post test. E. Inhibition of 11-KT-induced, zebrafish Ar-mediated transactivation of the MMTV promoter by flutamide. Cells were incubated for 24 h with increasing concentrations of 11-KT (1 pM to 10 µM) with or without 1 µM or 10 µM flutamide. Percentage (%) of response: values are given relative to the maximal amount of luciferase activity for each condition. Each point (in Figs. 3B, 3C and 3E.) represents the mean ± SEM, of three independent experiments. Curves were generated using non-linear regression (GraphPad Prism 4.0).

Localization of androgen receptor mRNA in zebrafish testis

To identify the cell types in zebrafish testis that express *ar* mRNA, we performed *in situ* hybridization on 10 µm thick cryosections. At low power magnification, a clear signal was observed in discrete cells scattered throughout the testis, in the sections that were hybridized with the antisense cRNA *ar* probe (Fig. 4A). No signal was observed with the sense cRNA *ar* probe (Fig. 4B), indicating the specificity of the antisense probe generated against the sequence of zebrafish *ar* mRNA. At a higher magnification (Fig. 4C), the *in situ* hybridization signal was observed in the cytoplasm of Sertoli cells, judged by the shape and intratubular position of the signal. The Sertoli cell is the only intratubular somatic cell type and differs from the germ cells by showing a triangular or kidney-shaped nucleus, in contrast to the round or oval nuclear shape of germ cells.

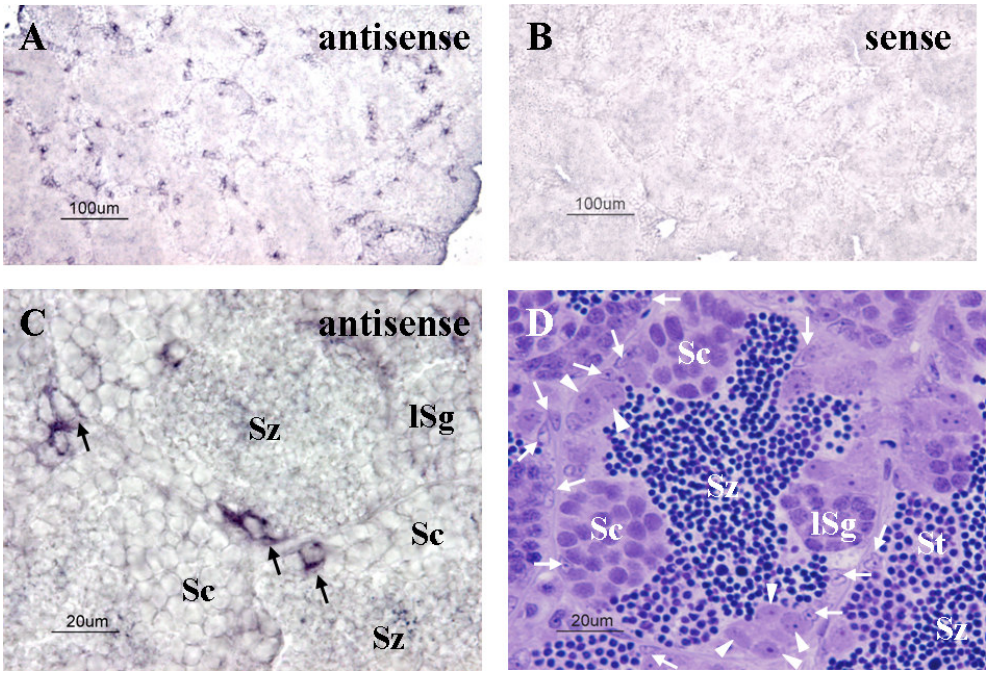

Figure 4 ***In situ*** **hybridization analysis of** ***ar*** **mRNA localization in zebrafish testis.** Lower magnification (10X) of a 10µm cryosection of zebrafish testis, hybridized with the *ar* antisense cRNA probe (A), showing signal in discrete cells, and hybridized with the *ar* sense cRNA probe (B), showing no aspecific signal. Higher magnification (60X) of a cryosection of zebrafish testis, hybridized with the *ar* antisense cRNA probe (C), revealed staining in the cytoplasm of a subpopulation of Sertoli cells. Resin-embedded histological section (4µm) stained with toluidine blue (D) shows the position of different germ cell stages and Sertoli cells. Indicated are the nuclei of Sertoli cells (arrows) and early spermatogonia (arrowheads), while late spermatogonia are indicated with lSg, spermatocytes by Sc, spermatids by St, and spermatozoa by Sz (in C and D). Scale bars are shown in each panel.

Not all Sertoli cells showed the same level of *ar* mRNA expression, since only a subset of Sertoli cells were stained (Fig. 4C), compared with the higher number of Sertoli cells lining a tubule in a histological section at the same magnification (see for comparison Fig. 4D). Based on the size, shape, number, and position close to the tubular basement membrane of the germ cells enveloped by the subpopulation of *ar* mRNA positive Sertoli cells, these germ cells were identified as early spermatogonia present as single cells or in small groups. No clear *in situ* hybridization signal was observed for peritubular myoid and interstitial Leydig cells (data not shown).

Discussion

This study describes the cDNA cloning and expression analysis (see Supplemental information) of the zebrafish *ar*. Moreover, we identified the physiologically relevant androgens, produced in the zebrafish testis, and included these – together with other steroids – in the functional characterization of the zebrafish Ar.

In the present study, a positive *ar in situ* hybridization signal was detected in a subpopulation of Sertoli cells in zebrafish testis. The majority of Sertoli cells, however, remained unlabeled, indicating that not all Sertoli cells have the same level of *ar* mRNA expression. Spermatogenesis in zebrafish, as in all other fish and amphibians, occurs within spermatogenic cysts. The cysts are formed when Sertoli cells envelope a germ cell clone by cytoplasmic extensions. Within each cyst, germ-cell development occurs synchronously and different cysts contain clonal lines of germ cells at different developmental stages (for review, see Schulz & Miura 2002). From our evaluation of the morphology and position of the germ cells surrounded by the zebrafish *ar* mRNA-positive subpopulation of Sertoli cells, we conclude that Sertoli cells in contact with early spermatogonia express the highest levels of *ar* mRNA in zebrafish testis. Interestingly, testicular explants from immature Japanese eel containing only early spermatogonia responded to incubations with 11-KT by showing full spermatogenesis (Miura *et al.* 1991), starting with several rounds of rapid proliferation of spermatogonia. A high level of expression of *ar* mRNA in Sertoli cells surrounding early spermatogonia would be consistent with the notion that these Sertoli cells are the target of stimulatory effects of 11-KT, resulting in a stimulation of spermatogonial proliferation and differentiation (Miura & Miura 2001). Likewise, the level of Ar protein in the zone of the salamander testis that contains predominantly spermatogonia was 1.5- to 5-fold higher than in zones containing further advanced germ cell types (Singh & Callard 1992). Future work has to demonstrate if 11-KT has similar effects on zebrafish spermatogenesis as in Japanese eel, and if progress of spermatogenesis

beyond the stage of early spermatogonia is associated with a down-regulation of *ar* mRNA levels in the Sertoli cells contacting later germ cell stages. Although in mammalian testis a particular Sertoli cell supports germ cells in different stages of development simultaneously, differences in *Ar* mRNA levels among Sertoli cells that depend on the stage of the seminiferous epithelial cycle have been described in rat (Shan *et al.* 1995). In the same study, *Ar* mRNA has been detected in Leydig cells and peritubular myoid cells, albeit at much lower levels than in Sertoli cells at adulthood. In the present study no prominent, positive *in situ* hybridization signal was found in somatic cell types other than Sertoli cells, indicating that the levels of *ar* mRNA in Leydig cells and peritubular myoid cells in zebrafish testis are too low to be detected by the present *in situ* hybridization approach.

Naturally occurring androgens as well as synthetic androgens are potential ligands for ARs. Our studies suggest that OHA, OA, and 11-KT are the major A2 metabolites of the adult zebrafish testis. Although we did not study the production of A2, it seems unlikely that this steroid is a quantitatively important end product of the zebrafish testis, *viz.* its rapid and effective conversion to OHA. Considering the low affinity and marginal transactivational capacity of OHA, it is unlikely to be a relevant AR ligand in zebrafish. However, OA and in particular 11-KT accumulate at the end of the biosynthetic chain and show, respectively, reasonable and high binding affinity and transactivation properties. We propose to consider 11-KT as the physiologically most important androgen of the group of 11-oxygenated steroids produced by zebrafish testis, in particular because 11-KT holds the most downstream position in the steroidogenic pathway. In closely related species, such as goldfish (*Carassius auratus*) (Abdullah & Kime 1994) or common carp (Barry *et al.* 1990), 11-oxygenated teleost androgens have been identified as main products of testicular steroidogenesis, such as 11-KT in goldfish, or OA in common carp, which can either be converted to 11-KT by 17β-hydroxysteroid dehydrogenase (Hsd17b) activity residing in erythrocytes of many fish species (Mayer *et al.* 1990), or is directly produced by carp testis tissue with an efficiency increasing during pubertal maturation (Consten *et al.* 2002), suggesting that an increasing Hsd17b activity (*i.e.* conversion of OA to 11-KT) is one of the factors associated with puberty. A testicular *hsd17b* type 3 cDNA has been identified recently in zebrafish (Mindnich *et al.* 2005), which converted OA to 11-KT. Although the same enzyme also has the catalytic capacity to convert OHA to OHT and A2 to T when transfected into a cell line (Mindnich *et al.* 2005), these conversions are not occurring to a noteworthy degree in zebrafish testis tissue fragments. A possible explanation may be competition for the substrates in the primary tissue culture: we showed that A2 is rapidly converted to OHA by

Cyp11b activity, possibly restricting the A2 to T conversion, while Hsd17b-mediated conversion of OHA to OHT may be hampered by the Hsd11b-catalyzed conversion of OHA to OA.

The very low levels of T production in zebrafish testis tissue may seem surprising, also considering that circulating levels of T reach ca. 50% of those of 11-KT in adult male carp (Koldras *et al.* 1990). However, a similar situation has been described in African catfish where the testicular production of T is at least 200-fold lower than the one of 11-oxygenated androgens (Vermeulen *et al.* 1994), while T plasma levels are in the same order of magnitude as 11-KT (Schulz *et al.* 1994). The possibility that circulating T might be derived from extra-testicular sources was excluded for the catfish (Vermeulen *et al.* 1994) since castration decreased T plasma levels below the detection limits. We therefore speculate that the relatively high T plasma levels reflect the high affinity and high capacity binding of T to sex steroid-binding globulin (SBG), protecting T from rapid breakdown and thereby prolonging its biological half-life time. An SBG-like protein has been identified in zebrafish (Miguel-Queralt *et al.* 2004), and steroid binding characteristics have been studied in a number of species, including the close zebrafish relatives goldfish (Pasmanik & Callard 1986) and carp (Chang & Lee 1992), showing that T (and E_2) but not 11-KT are bound with high affinity and capacity.

While no information on circulating androgens is available in zebrafish, respective data have been published from closely related, bigger species. In common carp, 11-KT and T were quantified at different stages of the reproductive cycle, and the concentrations varied between 3-6 and 1.5-2.5 ng/ml, respectively (Koldras *et al.* 1990). In goldfish (Rosenblum *et al.* 1985), 11-KT and T levels varied at different stages of testis development between 0.5-8.5 and 0.6-10, ng/ml, respectively. Taking the above consideration and our pharmacological and steroidogenesis data, we conclude that 11-KT is likely to be the main androgen in adult male zebrafish, while T may fulfil specific roles as well. As regards the K_d, K_i, and EC_{50} values for 11-KT in the range of 2-5 nM (see below), the plasma concentrations of 11-KT ranging from 0.5 to 10 ng/ml – *i.e.* 1.5-30 nM – in male carp and goldfish, would be well suited to activate the Ar in zebrafish.

We have shown that the zebrafish Ar is a functional AR, which is supported by high affinity androgen binding and androgen-dependent transactivational capacity. Comparison of the ligand-binding and transactivation properties of the zebrafish Ar revealed that steroids with a high affinity for the receptor (*i.e.* MT, MB, DHT, T, OHH_2P and 11-KT) also gave a high induction of

zebrafish Ar-mediated transactivation. The exception is OHH_2P, which could only induce transactivation in the high nanomolar range.

Similar binding affinities for the zebrafish Ar have been obtained by Jørgensen *et al.* (2007) for DHT, 11-KT, T and A2. In the regard of high affinity binding to synthetic androgens as well as 11-KT, T and DHT, the zebrafish Ar protein is similar to a number of other piscine Ar proteins cloned from rainbow trout (*i.e.* Ara) (Takeo & Yamashita 2000), fathead minnow (Wilson *et al.* 2004), and three-spined stickleback (Olsson *et al.* 2005). Studies on androgen binding to tissues extracts indicated that in some species two distinct patterns of androgen binding were found – one with rather specific binding of T, the other more similar to the broader specificity found for the zebrafish Ar in the present study. Binding of a broad range of synthetic and natural androgens, as found for the zebrafish Ar, was shared by one of the Ar types present in Atlantic croaker (Sperry & Thomas 1999) and coho salmon (Fitzpatrick *et al.* 1994) gonad tissue.

The transactivation properties of zebrafish Ar relate well to those of rainbow trout Ara, which did not distinguish between T and 11-KT (Takeo & Yamashita 2000). Transactivation studies with both Japanese eel Ar proteins (using a fixed concentration [100 nM] of the steroids tested) revealed that 11-KT, DHT, MB and MT were the most potent steroids in terms of transactivation of eel Ara (Todo *et al.* 1999), and 11-KT, MB and MT of eel Arb (Ikeuchi *et al.* 1999).

Data on zebrafish Ar transactivation, but not on receptor binding, have been reported very recently (Hossain *et al.* 2008) with regard to five androgens we have studied as well, however, using a zebrafish liver cell line. While similar data have been obtained as regards the two main androgens (11-KT and T), EC_{50} values for MT and DHT were reported to be ~100-fold lower than the results presented here, while A2 that we found to have reasonable transactivation activity, was reported to be inactive. The relatively low activity of DHT and MT does not appear to be in line with studies that reported on the transactivation profiles of other fish Ar proteins (Ikeuchi *et al.* 1999; Todo *et al.* 1999; Takeo & Yamashita 2000). Moreover, the well-established use of MT as a compound to induce female-to-male sex reversal in zebrafish research (Westerfield 2000) or salmonid aquaculture (Donaldson & Hunter 1982) provides evidence for the biological activity of this compound. It also seems important to note that Hossain *et al.* (2008), when modeling the zebrafish Ar binding site to calculate interaction energy between Ar and different ligands, reported that DHT and A2 showed interaction energies similar to 11-KT.

The pharmacological characterization of Ar subtypes from the different species (Todo *et al.*, 1999; Ikeuchi *et al.*, 1999; Takeo & Yamashita 2000; Wilson *et*

al., 2004; Olsson *et al.*, 2005) does not show sufficient overlap to draw a general conclusion at present, because of differences in experimental set up (*i.e.* the use of different cell lines, different tracers, and different promoter-reporter constructs). Taken together, however, it seems that the zebrafish Ar described here groups well with Ar proteins characterized in fathead minnow, rainbow trout (*i.e.* Ara), Japanese eel (both Ara and Arb) and Atlantic croaker (type 2 Ar), presenting a broad androgen-binding specificity.

In summary, we found a single gene coding for a nuclear Ar in the zebrafish, and no indications exist for another *ar* gene in the zebrafish genome. A similar conclusion, supported by Southern blot analysis, has been drawn by Hossain *et al.* (2008). The zebrafish *ar* mRNA is expressed in all tissued examined and our *in situ* hybridization studies revealed that high levels of expression in adult testis are found in the subpopulation of Sertoli cells that contact early spermatogonia. Furthermore, the receptor has been characterized *in vitro* to respond well in terms of binding as well as transactivation to 11-KT and T, two natural androgens proposed to be the physiologically most relevant androgens in zebrafish. The pharmacological characteristics and the tissue-distribution pattern of the zebrafish Ar will allow us to further study the role of this receptor in male sex differentiation and spermatogenesis.

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Supplemental information

In this supplemental file, the full-length cDNA cloning of the zebrafish *ar*, the phylogenetic analysis of the zebrafish Ar, and the expression of the zebrafish *ar* mRNA in different tissues and during different developmental stages is described.

Material and Methods

RNA isolation and cDNA synthesis

Total RNA was extracted from various tissue samples from three male as well as three female zebrafish, from 63 zebrafish embryos (24 hours post fertilization; 24 hpf), and from testes and ovaries of 5 male and 5 female *vas::EGFP* transgenic zebrafish (Krøvel & Olsen 2002) at 56 days post fertilization (56 dpf) using the FastRNA Pro Green kit (MP Biomedicals, Solon, OH, USA) with minor modifications to the manufacturer's protocol. Before RNA isolation, the weight of each organ derived from an adult zebrafish was determined as well as the weight of the sample of the tissue that was used for total RNA extraction. In the RNA isolation procedure from zebrafish embryos, and from testes and ovaries of *vas::EGFP* transgenic zebrafish, the coprecipitant GlycoBlue (Ambion, Austin, TX, USA) was added to the water phase to a final concentration of 50 µg/ml before isopropanol precipitation.

Zebrafish testis total RNA was reverse transcribed with random hexamers using the Superscript II pre-amplification system (Invitrogen, Breda, The Netherlands), according to the instructions of the manufacturer.

PCR and RACE

Zebrafish testis total RNA was reverse transcribed with random hexamers using the Superscript II pre-amplification system (Invitrogen, Breda, The Netherlands), according to the instructions of the manufacturer. Poly(A)-rich zebrafish testis RNA was prepared using Dynabeads-oligo dT_{25} (Dynal A.S., Oslo, Norway), according to the manufacturer's instructions. Zebrafish poly(A)-rich testis RNA was reverse transcribed to 5'- and 3'-RACE ready cDNA using a SMART RACE cDNA amplification kit (Clontech, Mountain View, CA, USA), according to the manufacturer's instructions.

To obtain a partial zebrafish *ar* cDNA sequence, 2 µl random-primed zebrafish testis cDNA was used as template in a PCR amplification with degenerate primers 711 and 712 (Supplemental Table 1), corresponding to highly conserved amino acid sequences (TCGSCKVFF and KWAKGLPGF) found in

known ARs. PCR was carried out in a 50 µl volume, containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.01% gelatin, 200 µM each dNTP, 50 pmol primers in a Perkin-Elmer 2400 thermal cycler (Applied Biosystems, Foster City, CA, USA), using 1 unit Super*Taq* (HT Biotechnologies Ltd., Cambridge, Cambs, U.K.). PCR was performed under the following cycling conditions: denaturation at 94°C for 30 s, followed by 35 cycles of 94°C for 15 s, 59°C for 15 s and 72°C for 45 s. DNA fragments of approximately 440 bp were subcloned into pcDNA3.1/V5-His TOPO vector (Invitrogen, Breda, The Netherlands); plasmid DNA of 10 clones was prepared for DNA sequence analysis.

Supplemental Table 1 Primers used in this study

Primer	Nucleotide sequence (5′ → 3′)
711[a]	ACCTGYGGYAGCTGCAARGTKTTYTT
712[a]	AAAACCTGGCAWKCCYTTKGCCCAYTT
886	ACCCTGGGAGCCCGCAAGCTGAGGA
887	CGAGTGGAAAGTCAGGTTGGGCTTGG
962	TGAAAGGTCCGGATGAGGTCGGAG
963	ACAAGCACTGGACAGTCTCCGACG
1016	CCTCGGAGCTCTTCGCCTCTGTCTCGTCCC
1017	GTACTCTCGGTCTTTCCTTCCTGCCTCTCGTTCGCTC
1943[b]	TAGCCACCATGGAGGTTCCGGTCGGGCTG
1944[c]	GGTGTGGTATGGGGCGTGGTCCTGTCATTTGTGG
2430[d]	T3Rpps-CGAGTAGTAGTCGCGCGTGCA
2431[e]	T7Rpps-TCGTCACCTCACCAGCTAGCTGTTCCA

[a]Primers 711 and 712 are degenerate primers; Y = T or C, R = G or A, K = G or T and W = A or T.
[b]Primer 1943 contains a consensus translation initiation sequence (Kozak 1984; underlined) at its 5′-end.
[c]Primer 1944 contains 10 unrelated nucleotides at its 5′-end, which are underlined.
[d]Primer 2430 contains the T3 RNA polymerase promoter sequence (underlined) at its 5′-end (T3Rpps; 5′-GGGCGGGTGTTATTAACCCTCACTAAAGGG-3′).
[e]Primer 2431 contains the T7 RNA polymerase promoter sequence (underlined) at its 5′-end (T7Rpps; 5′-CCGGGGGGTGTAATACGACTCACTATAGGG-3′).

To isolate the 5′- and 3′-ends of the zebrafish *ar* cDNA, gene-specific primers (*i.e.* primers 887 and 886; Supplemental Table 1) – based on the consensus nucleotide sequence of the 10 clones, each containing the above mentioned 440 bp PCR product – 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 (*i.e.* primers

963 and 962; Supplemental Table 1) in combination with a nested universal primer (NUP), respectively. To obtain more sequence information at the 5'-end of the zebrafish *ar*, an additional 5'-RACE amplification was performed using the gene-specific primers 1016 and 1017 in combination with UPM and NUP, respectively. 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), using Advantage 2 polymerase (Clontech). RACE products were subcloned into pcDNA3.1/V5-His TOPO vector (Invitrogen). DNA sequence analyses were performed on automated ABI PRISM 310 and 377 DNA sequencers, using Dye Terminator cycle sequencing chemistry (all from Applied Biosystems).

DNA sequence analysis and phylogenetic analysis

A homology search was performed using the BLAST program (Altschul *et al.* 1990). The alignment of multiple protein sequences, and the calculations of the percentages similarity and percentages divergence, were performed using the Megalign program of the Lasergene software package (DNASTAR Inc., Madison, WI, USA) with the Clustal (PAM250) algorithm (Higgins & Sharp 1989). The percentage similarity is a measure of similarity between a given AR sequence and the zebrafish Ar sequence, derived by taking the matches over the matches, mismatches and gaps, according to the formula: *similarity = (100 x consensus length) / (consensus length + mismatches + gaps)*. The percentage divergence is a measure of how far a particular AR sequence in the set has diverged form the zebrafish Ar sequence. It is derived by taking its distance from the ancestral node over the total length of the tree, according to the formula: *divergence = sum (residue distances) + (gaps x gaps penalty) + (gap residue x gap length penalty)*. The following full-length (deduced) amino acid sequences were used: *Danio rerio* Ar (EF153102; this study), *Acanthopagrus schlegelii* Ar (AAO61694), *Anguilla anguilla* Ara (BAA75464), *Anguilla anguilla* Arb (BAA83805), *Astatotilapia burtoni* Arb (AAL92878), *Canis familiaris* AR (AAQ84563), *Carassius auratus* Ar (AAM09278), *Crocuta crocuta* AR (Q8MIK0), *Dicentrarchus labrax* Ar (AAT76433), *Eulemur fulvus collaris* AR (O97776), *Gallus gallus* AR (NP_001035179), *Gambusia affinis* Ara (BAD81045), *Gambusia affinis* Arb (BAD51046), *Gasterosteus aculeatus* Arb1 (AAO83573), *Gasterosteus aculeatus* Arb2 (AAO83572), *Halichoeres trimaculatus* Ar (AF326200), *Homo sapiens* AR(1) (AAI32976), *Homo sapiens* AR(2) (AAA51770), *Kryptolebias marmoratus* Ar (ABC68612), *Macaca fascicularis* AR (O97952), *Micropogonias undulates* Ar (AAU09477), *Mus musculus* AR (NP_038504), *Oncorhynchus mykiss* Arb

(BAA32785), *Oncorhynchus mykiss* Ara (BAA32784), *Oreochromis niloticus* Ara (BAB20081), *Oreochromis niloticus* Arb (BAB20082), *Oryzias latipes* Ara (BAC98301), *Pagrus major* Ar (BAA33451), *Papio hamadryas* AR (O97960), *Pimephales promelas* Ar (AAF88138), *Rana catesbeiana* Ar (Q7T1K4), *Rattus norvegicus* AR (AAA40759), *Saimiri boliviensis* AR (ABO64644), *Sus scrofa* AR (AAG37994), *Tetraodon nigroviridis* Ar (CAG02975), *Xenopus laevis* Ar (AAC97386), while the following progesterone receptor (PR; Pr) and estrogen receptor (Esr) sequences were used as outgroups: *Anguilla anguilla* Pr (BAA89539), *Danio rerio* Pr (AAY85275), *Danio rerio* Esr1 (NP_694491), *Danio rerio* Esr2a (NP_851297) and *Danio rerio* Esr2b (NP_777287), *Homo sapiens* PR (AAD01587), *Mus musculus* PR (NP_032855), *Xenopus laevis* Pr (AF279335). A phylogenetic tree was constructed from the aligned sequences using the neighbor-joining method (Saitou & Nei 1987).

Analysis of zebrafish androgen receptor mRNA tissue distribution by real-time, quantitative PCR

Primers to detect zebrafish *ar* mRNA (Supplemental Table 2), and primers and fluorogenic probe to detect the endogenous control RNA (zebrafish elongation factor 1α [*ef1a*] mRNA; Supplemental Table 2), were designed. The specificity of the *ar* primer set, and the *ef1a* primers and probe set, were tested with real-time, quantitative PCR (qPCR) on serial dilutions of zebrafish testis cDNA, including a dissociation curve for the *ar* primers. All qPCRs for the *ar* were performed in 20 µl reactions, containing 10 µl Power SYBR Green PCR Master mix (Applied Biosystems, Foster City, CA, USA), 0.9 µM forward primer (*ar*-Fw), 0.9 µM reverse primer (*ar*-Rv) and 5 µl cDNA. All qPCRs for the *ef1a* were performed in 20 µl reactions containing 10 µl TaqMan Universal PCR Master mix (Applied Biosystems), 0.3 µM forward primer (*ef1a*-Fw), 0.3 µM reverse primer (*ef1a*-Rv), 0.1 µM TaqMan probe (*ef1a*-Pr) and 5 µl cDNA. Relative mRNA levels were determined using the 7900HT Fast Real-Time PCR System (Applied Biosystems), using default settings.

The relative *ar* mRNA levels in different tissues in adult zebrafish were calculated in a similar way as described previously (Bogerd *et al.* 2001), using the *ef1a* data for normalization. However, to present changes in *ar* mRNA levels between different tissues on the basis of absolute amounts per tissue, the adjusted relative *ar* mRNA level per tissue was calculated, using the following formula (adapted from Kusakabe *et al.* 2006):

Adjusted relative *ar* mRNA level$_{(tissue)}$ = {(relative *ar* mRNA level$_{(tissue)}$) x (total RNA amount after RNA extraction/tissue weight of tissue before RNA extraction)} / relative *ar* mRNA level$_{(testis)}$

Supplemental Table 2 Primers and TaqMan fluorogenic probes[a].

Target	GenBank accession number	Primers	Nucleotide sequence (5′ → 3′)
ar	EF153102	*ar*-Fw	ACGTGCCTGGCGTGAAAA
		ar-Rv	CAAACCTGCCATCCGTGAAC
ef1a	AF323692	*ef1a*-Fw	GCCGTCCCACCGACAAG
		ef1a-Rv	CCACACGACCCACAGGTACAG
		ef1a-Pr	CTCCAATTTTGTACACATCCTGAAGTGGCA

[a]Sequences are shown for the sense (-Fw) and antisense (-Rv) primers and/or the TaqMan probe (-Pr).

Results

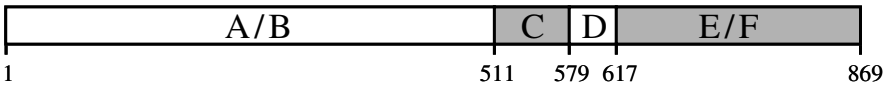
Zebrafish androgen receptor cDNA cloning

As a first step to isolate the zebrafish *ar* cDNA, we designed a set of degenerate primers (*i.e.* primers 711 and 712; Supplemental Table 1) homologous to highly conserved AR amino acid sequences. These primers were then used to PCR amplify an internal fragment of the zebrafish *ar*. A 444 bp PCR product was gel purified, extracted and cloned. The DNA sequences of the inserts of 10 clones were determined, and four new zebrafish *ar*-specific primers (*i.e.* primers 886, 887, 962 and 963; Supplemental Table 1) were designed based on the single consensus nucleotide sequence of the cloned RT-PCR products, and used in RACE reactions in order to isolate the 5′-end (primer 887 and nested primer 963) and 3′-end (primer 886 and nested primer 962) of the zebrafish *ar* cDNA. Because the 5′-RACE yielded PCR products too short to encode the full 5′-coding region of the zebrafish *ar* cDNA, additional primers (*i.e.* primers 1016 and 1017; Supplemental Table 1) were designed and used for a next round of 5′-RACE. The combination of the 5′-RACE and 3′-RACE reaction products yielded a 3625 bp cDNA sequence consisting of an open-reading frame of 2610 nucleotides that was flanked by leader

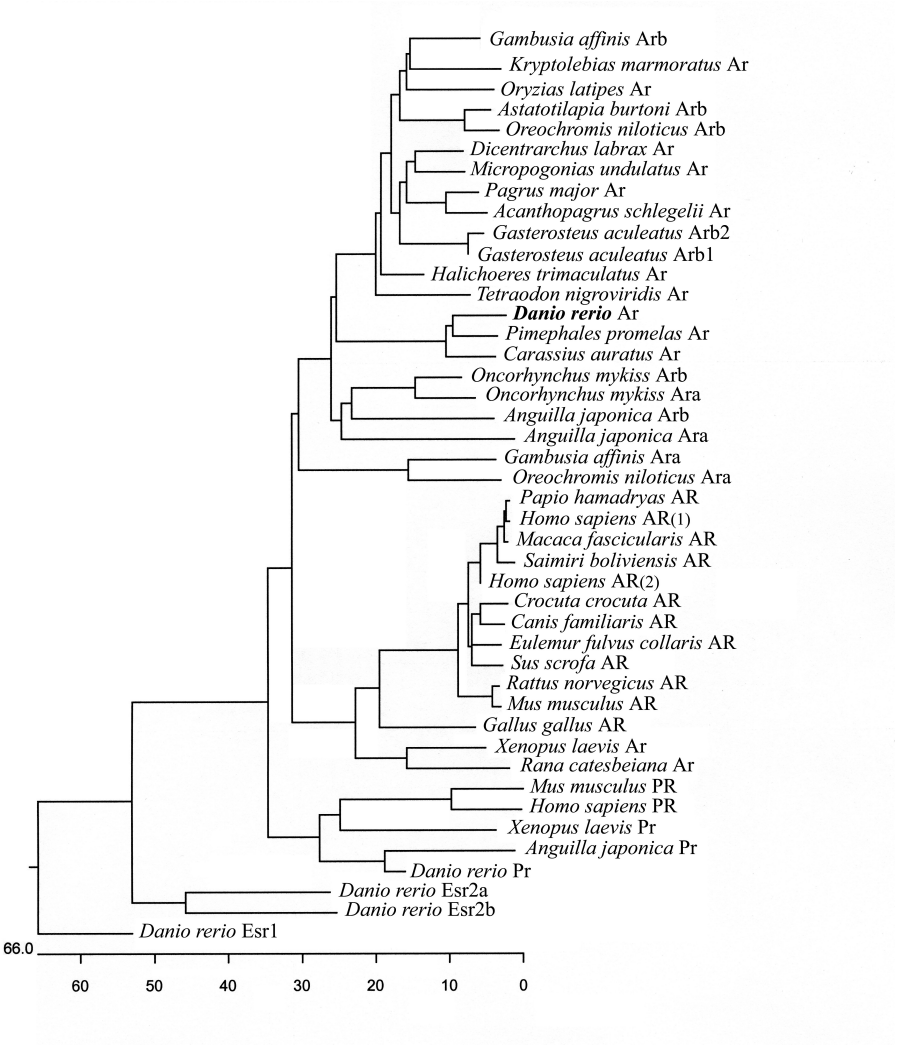
and trailer sequences of 537 and 478 nucleotides, respectively. The zebrafish *ar* open-reading frame of 2610 nucleotides was PCR amplified using primers 1943 and 1944, and subcloned into the pcDNA3.1/V5-His-TOPO expression vector in the correct orientation. Sequence analysis of several clones revealed identical sequences as those obtained in the 5'- and 3'-RACE clones. The nucleotide sequence of the zebrafish *ar* open-reading frame was deposited at GenBank with accession number EF153102.

A putative translation initiation codon was identified starting at nucleotide position 538; conceptual translation of the open-reading frame following this initiation codon predicted an 869-amino acid protein. The protein displayed the typical features and domain structure of members of the classical nuclear receptor family, and in particular of ARs (Supplemental Fig. 1); the protein has an additional threonine residue at position 180, and proline residues instead of glutamine and histidine residues on positions 213 and 467, respectively, compared with the proteins deduced from the *ar* cDNAs cloned by Jørgensen *et al.* (2007) and Hossain *et al*. (2008).

Amino acid sequence alignment of the zebrafish Ar with other AR proteins was performed using the Clustal method (Higgins & Sharp 1989). The zebrafish Ar had the highest amino acid identity with Ar proteins of fish species belonging to the *Cyprinidae*, followed by Ar proteins of other teleost fish species and other vertebrate species (Supplemental Table 3). A phylogenetic tree, constructed from the aligned amino acid sequences using the neighbor-joining method (Saitou & Nei 1987), revealed that the known ARs are divided into two major clades, whereas a selection of progesterone and estrogen receptors (that were used as outgroups) were divided into separate clades (Supplemental Fig. 2). One AR clade consisted of fish Ar proteins, while the other AR clade contained the mammalian, amphibian and avian AR proteins. Within the clade of fish Ar proteins, several groups could be recognized. The group that includes the zebrafish Ar (*i.e.* Ar proteins derived from cyprinid species) is closer to the β clade than to the α clade, in particular when the ligand binding domain is used in the phylogenetic analysis (Hossain *et al.* 2008) (Supplemental Fig. 2).



Supplemental Figure 1 Specific domains of the zebrafish androgen receptor. Schematic representation of the zebrafish Ar and the localization of specific domains; A/B domain, transactivation domain; C domain, DNA-binding domain; D domain, hinge domain; E/F domain, ligand-binding domain.



Supplemental Figure 2 Phylogenetic tree of ARs. The Clustal method was used to perform multiple sequence alignment. The phylogenetic tree was constructed using the Megalign program of the Lasergene software package (DNASTAR Inc.). The horizontal distances to the branching points are proportional to the number of amino acid substitutions.

Supplemental Table 3 Percentage similarity (indicated in bold) and percentage divergence (between brackets) of (domains of) the zebrafish Ar to (domains of) selected ARs. N.D., not determined; the analyses for these sequences could not be performed, since the *Halichoeres trimaculatus* Ar and *Danio rerio* Pr sequences are partial (*i.e.* lacking enough sequence information at the N-terminal end) to allow calculations.

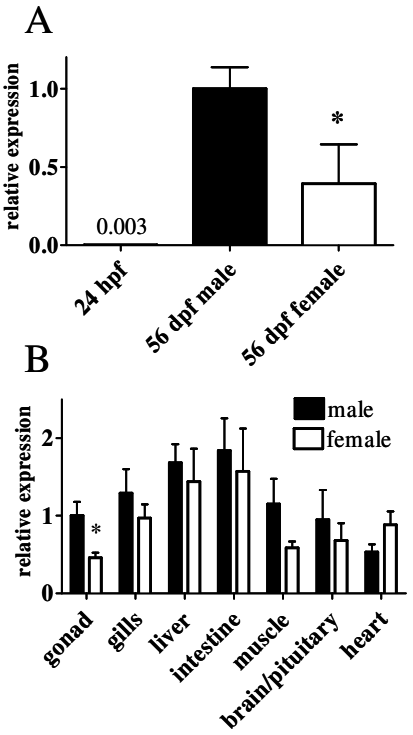
	Full length	A/B domain	C domain	D domain	E/F domain
Carassius auratus Ar	**83.0** (13.0)	**73.5** (20.3)	**97.1** (2.9)	**89.5** (10.5)	**96.0** (4.0)
Pimephales promelas Ar	**83.2** (13.2)	**74.0** (19.6)	**95.6** (4.4)	**86.8** (13.2)	**96.8** (3.2)
Halichoeres trimaculatus Ar	**62.5** (37.4)	**N.D.** (N.D.)	**95.6** (4.4)	**52.6** (47.4)	**82.2** (17.8)
Kryptolebias marmoratus Ar	**52.0** (45.1)	**19.8** (69.0)	**97.1** (2.9)	**50.0** (50.0)	**85.8** (14.2)
Oncorhynchus mykiss Arb	**51.6** (44.4)	**28.7** (57.9)	**95.6** (4.4)	**57.9** (42.1)	**88.1** (11.9)
Tetraodon nigroviridis Ar	**51.0** (43.7)	**18.1** (72.4)	**95.6** (4.4)	**51.4** (40.5)	**83.7** (16.3)
Anguilla japonica Arb	**50.9** (47.3)	**24.4** (67.3)	**95.6** (4.4)	**56.8** (35.1)	**84.2** (15.8)
Gambusia affinis Arb	**50.6** (47.8)	**22.2** (70.9)	**97.1** (2.9)	**47.4** (52.6)	**84.2** (15.8)
Oncorhynchus mykiss Ara	**50.4** (46.4)	**30.5** (58.8)	**92.6** (7.4)	**52.6** (47.4)	**86.2** (13.8)
Pagrus major Ar	**49.5** (47.6)	**20.5** (71.6)	**94.1** (5.9)	**55.3** (44.7)	**85.0** (15.0)
Oreochromis niloticus Arb	**49.0** (48.4)	**20.5** (72.3)	**97.1** (2.9)	**52.6** (47.4)	**82.2** (17.8)
Anguilla japonica Ara	**44.6** (52.0)	**20.0** (70.5)	**92.6** (7.4)	**54.1** (43.2)	**77.9** (43.2)
Gambusia affinis Ara	**41.6** (52.5)	**16.1** (76.5)	**80.9** (19.1)	**21.1** (80.0)	**69.6** (30.0)
Gallus gallus AR	**40.4** (56.6)	**12.6** (79.2)	**91.2** (8.8)	**40.5** (54.1)	**64.8** (31.9)
Oreochromis niloticus Ara	**39.4** (54.5)	**13.5** (76.6)	**80.9** (19.1)	**18.4** (81.6)	**65.6** (32.1)
Canis familiaris AR	**38.2** (62.9)	**17.2** (77.8)	**91.2** (8.8)	**37.8** (59.5)	**67.6** (29.9)
Homo sapiens AR (1)	**38.9** (62.3)	**17.8** (77.9)	**91.2** (8.8)	**37.8** (59.5)	**68.0** (29.5)
Rattus norvegicus AR	**38.9** (62.5)	**17.4** (76.4)	**91.2** (8.8)	**35.1** (59.5)	**68.0** (29.5)
Mus musculus AR	**38.3** (62.4)	**17.0** (76.0)	**91.2** (8.8)	**35.1** (59.5)	**68.0** (29.5)
Danio rerio Pr	**47.5** (48.7)	**N.D.** (N.D.)	**75.0** (25.0)	**21.1** (76.3)	**47.0** (50.2)
Danio rerio Esr1	**15.3** (71.6)	**14.6** (83.2)	**57.6** (42.4)	**15.8** (83.8)	**18.6** (72.9)

Ontogeny and tissue distribution of zebrafish androgen receptor mRNA

The presence of zebrafish *ar* mRNA was detected in all tissues examined using real-time, quantitative RT-PCR analysis (Supplemental Fig. 3). Zebrafish *ar* expression could already be determined in 24 hpf embryos, although this expression was approximately 300-fold lower compared with *ar* expression in 56 dpf zebrafish testis (Supplemental Fig. 3A). An approximately two- to two-and-a-half-fold lower *ar* expression was observed in 56 dpf zebrafish ovary relative to the situation in 56 dpf zebrafish testis (Supplemental Fig. 3A).

The expression levels of *ar* mRNA in various tissues of adult zebrafish (derived from males as well as females), were expressed relative to the levels in zebrafish testis (Supplemental Fig. 3B). In all tissues examined, except for heart, the relative *ar* expression had a tendency to be higher in males than in females, however *ar* was only significantly higher expressed in male gonad. Relative to *ar* expression in testis (Supplemental Fig. 3B), *ar* mRNA levels in liver and intestine

were slightly higher in both sexes, and approximately two-fold lower in ovary and muscle (in females) and heart (in males).



Supplemental Figure 3 Androgen receptor mRNA expression during ontogeny and tissue-distribution in zebrafish. A. Total RNA was extracted out of sixty three, 24 hpf zebrafish embryos and the gonads of previously sexed 56 dpf *vas::EGFP* transgenic zebrafish (n=5 for both testis and ovary; Krøvel and Olsen, 2002). Zebrafish *ar* mRNA levels were determined with real-time quantitative RT-PCR. Each column represents the mean expression of *ar* mRNA relative to zebrafish *elongation factor 1a (ef1a)* mRNA ± SEM normalized to the relative expression of *ar* mRNA in zebrafish testes. B. Total RNA (1.2 µg) was extracted from various tissues of zebrafish males (black columns) and females (white columns), including gills, testes and ovaries, liver, intestine, muscle, brain/pituitary and heart. Relative *ar* mRNA levels were determined with real-time, quantitative PCR. Each column represents the mean adjusted relative expression levels of *ar* mRNA normalized to *ef1a* mRNA ± SEM (n=3), normalized to *ar* mRNA levels in zebrafish testis and accounting for RNA recovery from total tissue mass. The asterisks in both A and B indicate a significant difference in relative expression between male and female ($P<0.05$), as validated by unpaired Student's t-test.

Discussion

The structural features of the cloned zebrafish *ar* cDNA suggest it encodes a member of the nuclear receptor family. Molecular evidence exists that teleost fish possess multiple androgen receptors, originating from duplication events of an ancestral *ar* gene in the teleost lineage, some time after the Actinopterygii diverged from the Sarcopterygii but before the teleost lineage (Wilson *et al.* 2004). Experimental trials to isolate additional cDNAs, which resemble zebrafish *ar,* as well as *in silico* approaches (*e.g.* searches of the *Danio rerio* ENSEMBL database [version 44.6e]; data not shown) to identify sequences, which are like zebrafish *ar*, did not provide evidence for the existence of additional *ar* forms in the zebrafish genome. A similar conclusion, supported by Southern blot analysis, has been drawn in a very recent paper (Hossain *et al.* 2008), in which the zebrafish *ar* sequence moreover was grouped in the β clade of the teleost Ar protein family.

The zebrafish Ar protein shares several structural features with Ar proteins from other teleost species. Our phylogenetic analysis placed the zebrafish *ar* sequence in a clade together with other Ar proteins from fish, and revealed that the zebrafish Ar has the highest similarity with two Ars identified in other cyprinid species (Wilson *et al.* 2004). This result is in accordance with the analysis of others (Jørgensen *et al.* 2007; Hossain *et al.* 2008) that were also able to identify two classes of teleost Ar proteins in their phylogenetic analysis. Our analysis clustered the zebrafish Ar in the β clade of teleost Ar proteins, similar to what has been found by Hossain et al. (2008). The existence of two classes of fish Ar is in accordance with the fact that two waves of gene duplication events have occurred in vertebrates (Laudet 1997); each type of Ar, identified in teleost fish that do express two types of Ar, is then present in a different class of receptors. Although also for rainbow trout two Ar proteins have been reported (Takeo & Yamashita 1999), it is assumed that these receptors are derived from tetraploidy as a result of a third gene duplication event in salmonids.

In zebrafish, *ar* mRNA was ubiquitously expressed in all tissues examined, usually with higher levels in male than in female tissues, except for the heart. The clear male-biased overexpression in gonads has also been reported by Hossain *et al*. 2008. These authors reported a male-biased overexpression in muscle. We also detected a higher *ar* mRNA expression in male muscle tissue, however, the difference with female muscle tissue was not statistically significant. This ubiquitous expression pattern of *ar* is in accordance with data obtained *ar* mRNAs in other species, for example during mouse and chicken embryogenesis (Crocoll *et al.* 1998; Katoh *et al.* 2006), and in adult rainbow trout (both *ar* genes) (Takeo & Yamashita 1999) and Japanese eel (predominantly *ara*) (Ikeuchi *et al.* 1999). In the latter species, however, *arb* mRNA was only detected in testis, muscle and spleen. In sea bass, *ar* mRNA levels were higher in testis compared to the levels found in ovary, brain, head kidney, liver, gill and spleen, regardless of sex (Blasquez & Piferrer 2005). Also in the hermaphrodite fish *Rivulus marmoratus*, *ar* mRNA levels were higher in gonads compared with other tissues (Seo *et al.* 2006).

Low levels of *ar* mRNA were already detected in zebrafish at 24 hpf in development, while these levels increased substantially in both male and female gonads – with approximately two-fold higher levels in testis – at 56 dpf. Zebrafish is a protogynous fish species, in which all individuals initially develop an ovary-like structure at ~14 dpf, irrespective of their final phenotypic sex (Wang *et al.* 2007). Final gonad differentiation takes place between 20 and 42 dpf and includes a juvenile 'ovary-to-testis' type transformation in male zebrafish (Wang *et al.* 2007). The higher *ar* mRNA level observed in testis compared with ovary at 56 dpf in this

study suggests that the Ar may have a functional role in this transformation process, as discussed recently by Hossain *et al*. 2008.

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3

Zebrafish primary testis tissue culture: An approach to study testis function *ex vivo*

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Abstract

To develop new tools to study the regulation of testis physiology in teleost fish, a medium-term *ex vivo* organ culture system was adopted for zebrafish testis tissue. The addition of 100 nM 11-ketotestosterone to the system supported complete spermatogenesis, as determined by morphological, molecular and immunohistochemical analyses. Under basal conditions, however, the development of differentiated spermatogonia, spermatocytes, and spermatids was seriously disturbed, probably related to the rapid (within 2 days) down-regulation of the steroidogenic system. Forskolin (0.5 μM) stimulated acute androgen release from freshly removed tissue and partially prevented down-regulation of the steroidogenic system. The present *ex vivo* culture system can serve as a tool to evaluate effects of a wide range of substances on the two main functions of the testis, spermatogenesis and hormone production.

Introduction

Complete spermatogenesis in *ex vivo* organ culture starting from undifferentiated spermatogonia has been reported in vertebrates to date only for Japanese eel (*Anguilla japonica*) (Miura et al., 1991). Certain steps of spermatogenesis have also been reported under organ culture conditions in salmonid fish (Amer et al., 2001) and amphibians (e.g., Yazawa et al., 2002). The cystic mode of spermatogenesis seen in both fish and amphibians is characterized by a less complex Sertoli-germ cell interaction than in amniote vertebrates, which may contribute to allow *ex vivo* spermatogenesis in medium-term (a few days to a few weeks) testis organ culture. Using dissociated testicular cell suspensions, proliferation and further development of differentiated spermatogonia or spermatocytes into spermatozoa has been reported for a number of fish species, such as medaka (*Oryzias latipes*; Saiki et al., 1997; Song and Gutzeit, 2003) and zebrafish (*Danio rerio*; Sakai, 2002). Using this approach, however, it is difficult to control the re-association process of somatic and germ cells, or feeder cells need to be introduced in the experimental system.

In order to develop new and relatively simple tools for studying the endocrine regulation of spermatogenesis in fish, the suitability of an organ culture system to support spermatogenetic development in testicular explants from sexually mature zebrafish was evaluated. We examined which steps of spermatogenesis took place under basal conditions or in the presence of 11-

ketotestosterone (11-KT), an androgen with well known relevance for fish spermatogenesis (Miura et al., 1991; Borg, 1994), as well as the functional state of the testicular steroidogenic system during the *ex vivo* organ culture.

Results and discussion

The size of zebrafish testis (~3-8 mg total weight) allowed that a whole (left or right) testis was incubated per culture well (Figs. 1A, 1B). Morphological evaluation indicated certain heterogeneity in the testicular cell composition among different individuals (Leal et al., 2008). Coefficients of variation among individual fish (n=9-11) were analysed for a number of molecular parameters, namely expression levels of the germ cells markers *ziwi* (expressed strongly in spermatogonia and weakly in spermatocytes; Houwing et al., 2007), *sycp3l* (spermatocytes; Yano et al., 2008), and *shippo1* (spermatids; Yano et al., 2008). Also, the expression of Leydig- and a Sertoli cells-specific genes (*rlx3c* [unpublished results] and *gsdf* [Sawatari et al., 2007], respectively), and the amounts of 11-KT released after 48 hours basal culture, were examined. The coefficients of variation ranged between 21 and 53% (mean 31%). Biological variation changed according to the parameter analysed, which may require adjustment of the sample size, depending on the choice of parameter(s) and the magnitude of the experimentally induced changes expected. Since morphological evaluation showed no apparent differences in germ cell composition between left and right testis (data not shown), we examined if the effect of inter-individual variation was minimized by incubating one testis under control and the other testis under experimental conditions. Our experiments used between 6 and 12 pairs of testis per condition. In this way, a consensus was achieved between ethical guidelines, which recommend minimizing the number of experimental animals, and keeping the variance acceptably low. Since the data obtained with this experimental design represent paired observations for each individual (*i.e.* basal *vs.* treated), statistical analysis was performed using paired t-tests. Using this procedure, no significant differences between left and right testis pairs (n=9-11) were identified for any of the above-mentioned parameters (Fig. 1C). This data set demonstrates the feasibility of our approach to randomly assign one of the two testes to control, the other to an experimental condition for trustworthy comparisons between groups (*i.e.* basal *vs.* treated) subjected to a specific *ex vivo* treatment. However, when comparing three or more groups, testis explants of different fish had to be used (*e.g.* time course experiments as shown in Fig. 3A), so that one-way ANOVA followed by a Student-Newman-Keuls test was used for statistical analysis.

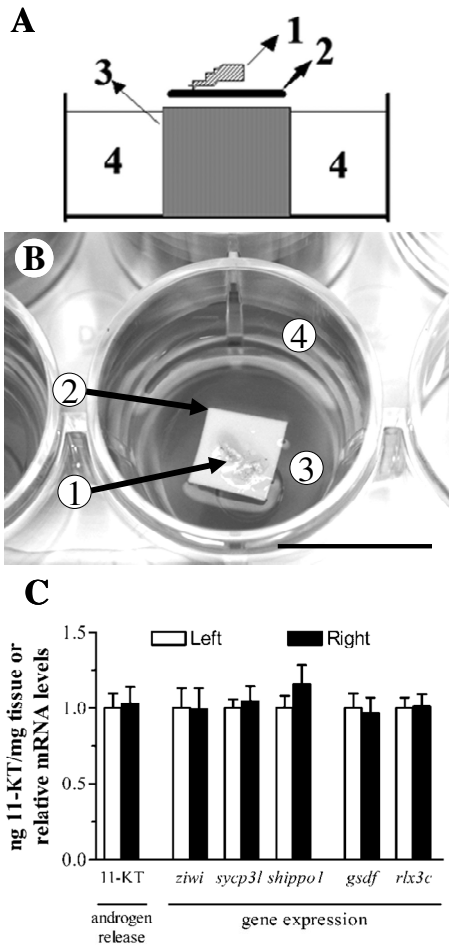

Fig. 1. (A) Schematic view and (B) photograph of the zebrafish testis organ culture system. 1, testis tissue; 2, nitrocellulose membrane; 3, agarose cylinder; 4, medium (1 ml). Scale bar: 1 cm. (C) Comparison of the *ex vivo* 11-KT release during 48 hours basal culture, and of mRNA levels of several germ and somatic cell markers (see text) between left and right testis for 9-11 individual fish. Data are represented as relative values normalised to the left testis. No significant differences between the two testes were found for any of the parameters analysed ($p > 0.05$).

We then addressed the capacity of the culture system to support spermatogenesis *ex vivo*. Freshly excised zebrafish testis tissue shows spermatogenic tubuli filled with sperm and spermatogenic cysts containing germ cells in all stages of spermatogenesis (Fig 2A). Spermatozoa were also abundant in testis sections taken from tissue after 7 days of culture under both basal and 11-KT-stimulated conditions. However, type B spermatogonia, spermatocytes and spermatids were much less numerous in tissue cultured in the absence of 11-KT (compare Figs. 2B and C and Suppl. Fig. 1A and B). The morphological observations were in accordance with significantly elevated *shippo1* mRNA levels (Fig. 2D). To investigate if 11-KT prevents the disappearance of pre-existing germ cells or stimulates germ cell proliferation and differentiation *ex vivo*, we exposed testis tissue explants to 5-bromo-2-deoxyuridine (BrdU, a DNA synthesis/proliferation

marker) during the first 48 hours of culture. Our results confirm the *ex vivo* progression of spermatogenesis under stimulation of 11-KT.

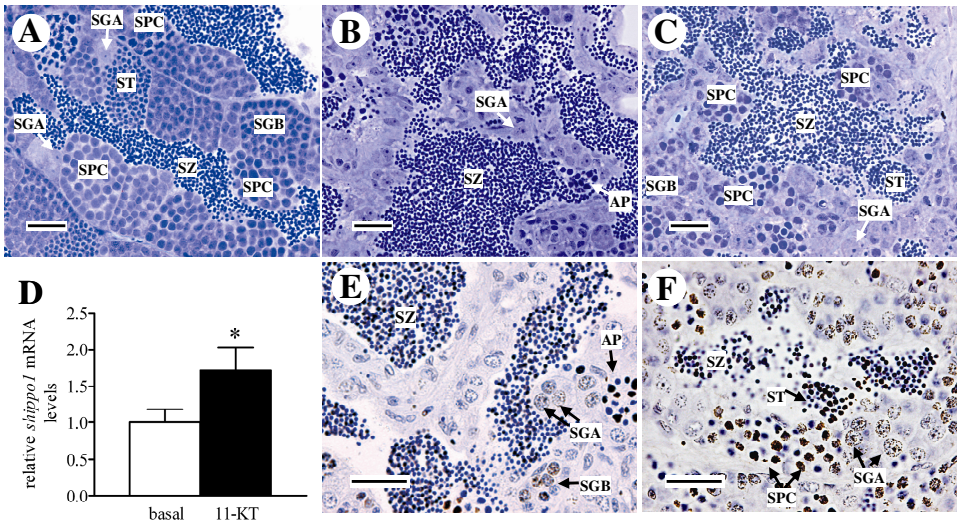

Fig. 2. Spermatogenesis in zebrafish testis after *ex vivo* organ culture. Freshly excised testis tissue showing all germ cell stages (A). Testis tissue was incubated under basal conditions (B) or in the presence of 100 nM 11-KT (C) for 7 days; higher numbers of B type spermatogonia (SGB), spermatocytes (SPC) and spermatids (ST) are present in the 11-KT treated explants. Apoptotic cells (AP), characterised by highly condensed nuclei, seem more abundant in explants cultured under basal conditions. SGA, type A spermatogonia; SZ, spermatozoa. (D) 11-KT exposure significantly elevates ($p<0.05$; $n=6$) *shippo1* mRNA levels. BrdU labelling of testis explant cultured under basal conditions (E) or in the presence of 11-KT (F). BrdU was present during the first two days of culture. Note that BrdU-labelling can be found in all germ cell stages. In all cases, the total length of the culture period was 7 days. Scale bars: 25 µm.

While BrdU-labelling was found in a limited number of spermatogonia, spermatocytes and apoptotic germ cells in tissue cultured under basal conditions (Fig. 2E, Suppl. Fig. 1A), BrdU-labelled germ cells were abundantly present in 11-KT-treated testis explants (Suppl. Fig. 1B); the higher magnification (Fig. 2F) revealed BrdU-labelling of spermatids and spermatozoa, demonstrating that the culture conditions allow completion of meiosis and spermiogenesis. The BrdU-labelling of type A and type B spermatogonia (Figs. 2E, 2F) demonstrates that they maintain their ability to proliferate and differentiate *ex vivo*. However, the system requires androgens to proceed to the rapid proliferative steps of spermatogonial differentiation and to enter meiosis. Sakai (2002) reported similar observations using dissociated testis cells, but feeder cells showing Sertoli cell characteristics were required to allow progression of spermatogenesis, suggesting that contact between germ and somatic cells is essential for zebrafish germ cell development in culture. With our *ex vivo* organ culture approach, the physiological relation between somatic and germ cells remains functionally preserved, as evidenced by

complete spermatogenesis under culture conditions. A primary tissue culture system allowing full spermatogenesis has been described previously for Japanese eel testis (Miura et al., 1991), which was the basis for developing the present system. Two important differences exist compared to the eel system: (1) the composition of the basal medium, the one used here being free of growth factors, and (2) the developmental stage of the testis tissue used; while we use here sexually mature testis containing all stages of germ cell development, the eel system uses immature testis containing only spermatogonia. While this makes the analysis of treatment effects more difficult in the zebrafish system, it provides a better opportunity for studying late(r) stages of, spermatogenesis.

The steroidogenic capacity was strongly down-regulated during the first 48 hours in basal culture conditions (Fig. 3). The most severe down-regulation (to ~8% within 6 hours of culture; Fig. 3A) was observed for *cyp17a1* mRNA levels. We consider it as likely that the decreased incidence of more differentiated germ cells, and perhaps also the increased incidence of apoptotic germ cells in tissue fragments incubated without 11-KT (see above), is a consequence of the decreasing capacity of the tissue to produce androgens. Nevertheless, testis tissue remained responsive (*i.e.* significant increase of 11-KT release over basal) to a short-term stimulation with 0.5 µM forskolin after up to 30 hours in basal culture, although the absolute 11-KT amount released decreased progressively (Fig. 3B). Despite the significant down-regulation of both *cyp17a1* and *star* mRNA levels after 48 hours, expression of both transcripts remained detectable even after 7 days of culture. Although this situation may maintain a low basal steroid production (Fig. 3A), it was insufficient for sustaining quantitatively normal spermatogenesis *ex vivo,* as suggested by the reduced number of type B spermatogonia, spermatocytes, and spermatids (Fig. 2A). From a practical point of view, the down-regulation of the steroidogenic system can be considered as advantageous, since it minimizes the interference of endogenous hormones with steroids added experimentally that, as shown for 11-KT, were able to promote spermatogenesis *ex vivo* (Fig. 2; Suppl. Fig. 1).

The continuous presence of 0.5 µM forskolin in the medium for 7 days partially prevented the down-regulation of the steroidogenic system observed in culture conditions (Fig. 3C). For example, the absolute 11-KT amount released into forskolin-containing medium was ~5 ng/mg testis tissue, 30% of which was released from culture day 3 to day 7 (complete culture environment was replaced at day 3). This demonstrates that the *ex vivo* culture system is able to support steroidogenesis for a medium-term period of time in the presence of an appropriate stimulating substance.

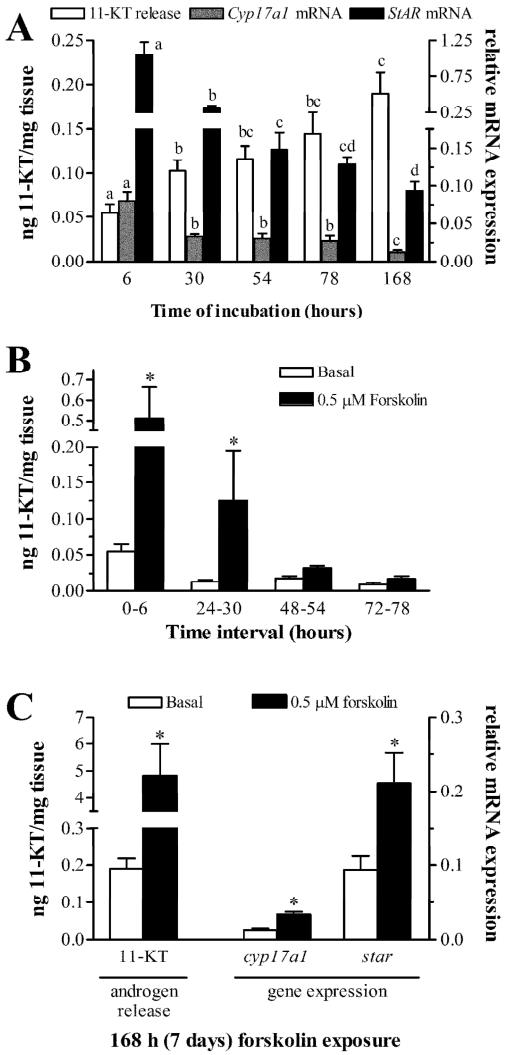

Fig. 3. Behaviour of the zebrafish testicular steroidogenic system under *ex vivo* organ culture conditions. (A) Time-course of 11-KT release and *cyp17a1* and *star* mRNA transcript levels in basal culture medium (n =6-7 at each time point; mRNA levels at t=0 hours is set to 1). (B) Forskolin-induced (0.5 µM) release of 11-KT during 6 hours of incubation commencing 0, 24, 48, or 72 hours after starting tissue culture in basal medium (n=6 per group). (C) Effects of continuous presence of 0.5 µM forskolin for 7 days on androgen release and testicular *cyp17a1* and *star* mRNA transcript levels (n=7). The cumulative androgen release is expressed as amount of 11-KT released per mg testis tissue incubated. mRNA expression levels are shown as relative values of respective transcript amounts measured in fresh testis tissue (n=12). For each parameter in panel (A), different letters denote statistical differences ($p<0.05$). Bars marked with an asterisk in panels (B) and (C) are significantly different from its respective basal condition control ($p<0.05$).

In summary, the current study supports the suitability, both from technical and biological points of view, of an *ex vivo* organ culture system to study zebrafish testis physiology. Such a system could serve as a powerful tool to evaluate the

effects of a wide range of substances, such as pituitary hormones, steroids, growth factors, as well as suspected endocrine disruptors, on many aspects of teleost fish spermatogenesis either at morphological, endocrine, or molecular level.

Material and methods

Animals

Sexually mature zebrafish, either Tübingen AB strain or outbred fish from a mixed background, were used. Animal culture (Westerfield, 2000) and experimentation were consistent with Dutch regulations and were approved by the Utrecht University Life Sciences Committee for Animal Care and Use.

Testis tissue culture technique

As a basis, we used a previously described organ culture system for Japanese eel testis (Miura et al., 1991). From each zebrafish, testes were removed from the body cavity and immersed for 2 min in 0.5% v/v commercial bleach in PBS containing 1 mM $CaCl_2$ and $MgCl_2$. After a 2 min buffer wash, the two testes were further incubated in parallel, one of them serving as control for the contra-lateral one. During culture, testis tissue explants were placed on of a 0.25 cm^2 nitrocellulose membrane (25 µm thickness, 0.22 µm pore size; Millipore, Billerica, MA,USA), itself resting on a 750 µl agarose cylinder (1.5% w/v in pH 7.4 Ringer's solution) placed in 1 ml medium in 24-well flat-bottom plates (Corning Inc., New York, USA) (Fig. 1A). Agarose cylinders were prepared using wells of 48-well flat-bottom plates (Corning Inc.) as moulds. Both membranes and cylinders were pre-soaked in basal medium overnight at 4°C. Basal culture medium consisted of Leibovitz-15 (Invitrogen, Carlsbad, CA, USA) supplemented with 10 mM HEPES (Merck, Darmstadt, Germany), 0.5% w/v bovine serum albumin fraction V (Roche, Mannheim, Germany), 10 nM retinoic acid (Sigma-Aldrich, St. Louis, MO, USA; see Supplemental information), 0.4 mg/L amphotericine B (Fungizone®; Invitrogen), 200 U/ml penicillin (Invitrogen), and 200 µg/ml streptomycin (Invitrogen); pH was adjusted to 7.4. Incubation was performed for 7 days in a humidified air atmosphere at 25°C; the medium was refreshed at day 3. For measuring androgen release by testicular tissue, the culture environment was refreshed by transferring the nitrocellulose membranes supporting the tissue explants to fresh culture wells.

Experimental validation of the culture technique

First, we evaluated if the two testes of one fish showed similar cellular compositions, mRNA levels of germ cell markers (*ziwi* for spermatogonia and spermatocytes, *sycp3l* for spermatocytes, and *shippo1* for spermatids) and somatic-cell-specific genes (*gsdf* for Sertoli cells and *rlx3c* for Leydig cells), and *ex vivo* 11-KT release after 48 hours of culture.

Second, the capacity of the culture system to support spermatogenesis was assessed by incubating testis tissue with 100 nM 11-KT. To some wells, 15 µg/ml BrdU (Sigma-Aldrich) was added during the first 48 hours in culture to evaluate germ cell proliferation and differentiation. After 7 days in culture, samples were processed for morphological evaluation, immunohistochemical detection of BrdU, or for analysis of mRNA levels of *shippo1*.

The functional state of the testicular steroidogenic system over culture time was studied in different ways. First, the time-course (6, 30, 54, 78, and 168 hrs) of 11-KT release and mRNA levels of two steroidogenesis-related genes (*cyp17a1* and *star*) was studied under basal culture conditions. Second, the responsiveness of testis tissue was quantified to an acute stimulation of 11-KT release for 6 hours with 0.5 µM of the adenylate cyclase activator forskolin (Sigma-Aldrich), starting after 0, 24, 48, or 72 hours in basal culture. Third, the effect of the continuous presence of 0.5 µM forskolin for 7 days on 11-KT release and testicular *cyp17a1* and *star* mRNA transcript levels was analysed. Pilot studies showed that 0.5 µM, forskolin elicited an intermediate stimulation of androgen release (unpublished data).

Miscellaneous techniques

Morphological evaluation was performed following previously reported procedures (Leal et al., 2008). BrdU-positive cells were detected following a modified protocol from van de Kant and de Rooij (1992) (see Supplemental information). Relative mRNA expression levels were determined by real-time, quantitative PCR as described previously (de Waal et al., 2008), except that the RNAqueous®-Micro Kit (Ambion, Austin, TX, USA) was used for total RNA extraction. Primers sequences and the detailed methodology for quantification of 11-KT release to the culture environment are given as Supplemental information.

Statistical analysis

Data are presented as mean ± SEM. Significant differences were identified using paired t-tests for two groups or one-way ANOVA followed by the Student-Newman-Keuls test for three or more groups. Significance level (p) was fixed at

0.05 in both cases.

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Supplemental information

We provide detailed methodological procedures for immunodetection of 5-bromo-2-deoxyuridine (BrdU)-positive cells in zebrafish testis tissue samples and for quantification of androgen release to the culture environment, as well as the sequences of the primers used to estimate relative mRNA expression levels by real time quantitative PCR (rt-qPCR). Moreover, an experiment on the effect of retinoic acid (RA) on spermatogenesis *ex vivo* is reported.

Immunohistochemistry

BrdU incorporation was immunodetected following a modified protocol from van de Kant and de Rooij (1992). Zebrafish testis tissue explants were fixed for 5 hours at room temperature in freshly prepared methacarn (60% [v/v] absolute ethanol, 30% chloroform, and 10% glacial acetic acid) and embedded in Technovit 7100 (Heraeus Kulzer, Wehrheim, Germany). Five µm thick sections were subjected to antigen retrieval (1% [v/v] periodic acid in water at 60°C for 30 min) and peroxidase blocking (1% [v/v] H_2O_2 in phosphate buffer saline [PBS] for 10 min). Thereafter, slides were incubated at room temperature for 1 hour with mouse anti-BrdU (BD Bioscience, San Jose, CA, USA; 1:80 diluted in PBS containing 1% [w/v] bovine serum albumin [BSA; Sigma-Aldrich, St. Louis, MO, USA]), and then for an additional hour with biotinylated horse anti-mouse (Vector, Burilingame, CA USA; 1:100 diluted in PBS containing 1% [w/v] BSA). Revelation of immunostaining was performed using avidin-biotin complex incubation for 1 hour (Vector) followed by DAB (Dako, Glostrup, Denmark) substrate development for 20 seconds. Nuclei were counterstained with haematoxylin Gills #3 (Sigma-Aldrich) for 30 seconds. For negative control, the primary antibody (i.e., mouse anti-BrdU) was replaced by the same concentration of normal mouse IgG (BD Bioscience).

Quantification of androgen release

At collection, testicular explants were weighed and incubation medium and agarose cylinders were transferred to a glass tube for homogenization (Ultra-turrax T25; Janke & Kunkel Ika-Labortechik, Staufen, Germany) and extraction with diethyl ether (four times with 5 ml solvent each). With this procedure, 11-ketotestosterone (11-KT) recovery was only 45% (n=6), although reconstitution of dry residues in 100 µl assay buffer and the high sensitivity of the radioimmunoassay used (3.5 pg per 50 µl; Schulz et al., 1994) allowed us to obtain measurable 11-KT quantities in all the samples analysed. Further recovery studies using tritiated 11-KT showed that 47% of total steroid added to the system could be

retrieved from the incubation medium exclusively (n=8), suggesting that combined extraction of incubation medium and homogenized agarose cylinder was only able to recover androgens present in the medium, but not those enclosed by agarose. The results were corrected for recovery efficiency (45 %) being expressed as amounts of 11-KT released per unit testis weight incubated.

Primer sequences for rt-qPCR analysis

Primers and 6-carboxy-fluorescein labelled probe were acquired to detect the endogenous control RNA, human eukaryotic 18S ribosomal RNA (TaqMan® gene expression assays; Applied Biosystems, Foster City, CA, USA). Specific primers to detect zebrafish *ziwi*, *sycp3l*, *shippo1*, *gsdf*, *rlx3c*, *cyp17a1*, and *star* were designed for their use in Sybr® green gene expression assays (Applied Biosystems). The specificity and amplification efficiency of each primer combination was tested on serial dilutions of zebrafish testis cDNA (results not shown). Respective primer sequences are shown in Supplemental Table 1.

Supplemental Table 1, Primers used to quantify mRNA levels by rt-qPCR analysis in zebrafish testicular explants

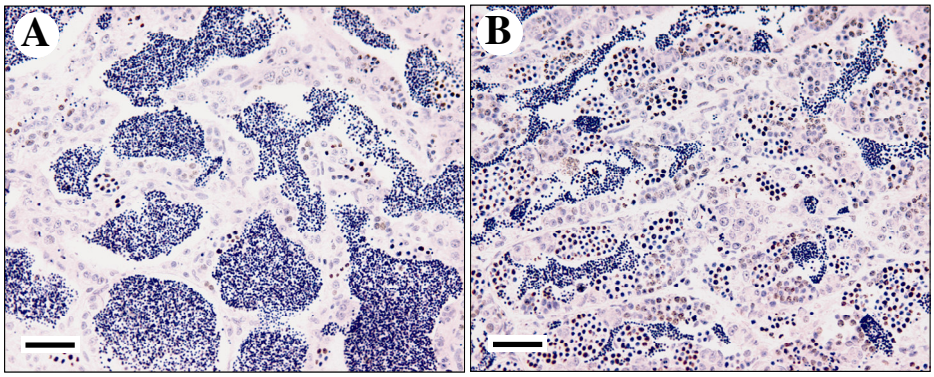
Target	Primer	Nucleotide sequence (5' → 3')
cyp17a1	Fw	GGGAGGCCACGGACTGTTA
	Rv	CCATGTGGAACTGTAGTCAGCAA
gsdf	Fw	CATCTGCGGGAGTCATTGAAA
	Rv	AGCTTGCCGGAGGACTCTG
rlx3c	Fw	TCGCATCGTGTGGGAGTTT
	Rv	TGGATAGAGACCTCGTTGTGCA
shippo1	Fw	GATGCCTGGAGACATGACCAA
	Rv	CAAAGGAGAAGCTGGGAGCTTT
star	Fw	CCTGGAATGCCTGAGCAGAA
	Rv	ATCTGCACTTGGTCGCATGAC
sycp3l	Fw	AGAAGCTGACCCAAGATCATTCC
	Rv	AGCTTCAGTTGCTGGCGAAA
ziwi	Fw	GATACCGCTGCTGGAAAAAGG
	Rv	TGGTTCTCCAAGTGTGTCTTGC

Sequences are shown for the sense (Fw) and antisense (Rv) primers.

Effect of RA in the culture medium

In the previously reported culture system for juvenile eel testis tissue (Miura et al., 1991), RA was present in the medium, a compound known to be required in rodents for the differentiation of spermatogonia (Van Pelt and De Rooij, 1991). For zebrafish spermatogenesis, however, a recent *in vivo* study showed that RA deficiency did not have negative effects on spermatogenesis while oogenesis was disturbed (Alsop et al., 2008). We tested if the absence or presence of 10 or 100 nM RA in incubation media had an influence on basal or 11-KT-supported (100 nM) spermatogenesis *ex vivo* after 7 days in culture. There was no difference in spermatogenic progress between testis tissue incubated in the absence or presence of 10 or 100 nM RA, both considering basal as well as 11-KT-stimulated conditions (data not shown), confirming the previously reported results for zebrafish spermatogenesis *in vivo* (Alsop et al., 2008). Nevertheless, 10 nM RA was kept as basal medium component for the sake of comparability with other culture systems and as regards use of the present culture medium for studies on ovarian functions.

Supplemental figure



Suppl. Fig. 1. Proliferation activity in *ex vivo* cultured zebrafish testis. BrdU labelling of testis tissue cultured under basal conditions (A) or in the presence of 100 nM 11-KT (B). BrdU was present during the first two days of culture. Note that BrdU-labelled germ cells are much more abundant in sections of 11-KT-treated testis explants. The tissue fragments were cultured for 7 days. Scale bars: 50 µm.

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4

Oestrogen-induced androgen insufficiency results in a reduction of proliferation and differentiation of spermatogonia in the zebrafish testis

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Abstract

Androgens can induce complete spermatogenesis in immature or prepubertal teleost fish. However, many aspects of the role of androgens in adult teleost spermatogenesis have remained elusive. Since oestrogens inhibit androgen synthesis, we used an oestrogen-induced androgen depletion model to identify androgen-dependent stages during adult zebrafish spermatogenesis. Exposure to 10 nM 17β-oestradiol *in vivo* at least halved the mass of differentiating germ cells (from type B spermatogonia to spermatids), while type A spermatogonia accumulated. Studies on the cellular dynamics revealed that a reduction of spermatogonial proliferation together with an inhibition of their differentiation to type B spermatogonia were the basis for the oestrogen-mediated disturbance of spermatogenesis. The capacity of the zebrafish testis to produce 11-ketotestosterone as well as the expression of steroidogenesis-related genes was markedly decreased after *in vivo* oestrogen exposure. Moreover, the androgen release response to recombinant zebrafish luteinizing hormone was lost after oestrogen exposure. We conclude that oestrogen exposure caused a state of androgen insufficiency in adult male zebrafish. Since the down-regulation of the steroidogenic system as well as disturbance of spermatogenesis in testicular explants exposed to 17β-oestradiol *ex vivo* was much less severe than after *in vivo* exposure, the main inhibitory effect appears to be exerted via feedback inhibition of gonadotropin release. This experimental set-up helped to identify spermatogonial proliferation and their differentiation as androgen targets in adult zebrafish spermatogenesis.

Introduction

Oestrogen signalling is involved in many processes in male reproduction, and is essential to achieve normal fertility, as indicated in mammals by studies conducted with oestrogen receptor (ER) knockout mice (Eddy *et al.* 1996; Hess *et al.* 2000; Akingbemi *et al.* 2003; Gould *et al.* 2007). Furthermore, oestrogenic feedback on the hypothalamus and pituitary controls the synthesis and release of gonadotropins in mammals, and hence the testicular synthesis of androgens (Jong *et al.* 1975; Lindzey *et al.* 1998; Turner *et al.* 2000). Leydig cells express ER (Zhou *et al.* 2002), and direct oestrogenic inhibition of steroidogenesis has been reported as well (Bartke *et al.* 1977).

Three types of ER exist in fish, namely ERα, ERβ1 and ERβ2 (Hawkins *et al.* 2000; Menuet *et al.* 2002; Choi & Habibi 2003; Halm *et al.* 2004; Filby & Tyler 2005); the types are designated as (*gene*/protein) *esr1*/Esr1, *esr2b*/Esr2b and *esr2a*/Esr2a, respectively, following the Official Zebrafish Nomenclature Guidelines (http://zfin.org). All types of ER are expressed in testis (Hawkins *et al.* 2000; Menuet *et al.* 2002; Choi & Habibi 2003; Halm *et al.* 2004; Filby & Tyler 2005). Studies describing their cellular localisation in testis indicate a heterogeneous pattern of mRNA and protein expression; for example, Esr1 protein has been found in the interstitial tissue of rainbow trout (*Oncorhynchus mykiss*) testis (Bouma & Nagler 2001), *esr1* and *esr2* mRNA expression were found in Japanese common goby (*Acanthogobius flavimanus*) and Japanese eel (*Anguilla japonica*) Sertoli cells (Miura *et al.* 1999; Ito *et al.* 2007), while Esr1 and Esr2 proteins were produced by meiotic and post-meiotic germ cells in channel catfish (*Ictalurus punctatus*) testis (Wu *et al.* 2001). Functionally, oestrogens stimulate spermatogonial stem cell renewal in immature Japanese eel testis probably via Sertoli cells (Miura *et al.* 1999; 2003). In amphibians, which have a cystic organization of spermatogenesis similar to fish, oestrogens promote proliferation of spermatogonia (Minucci *et al.* 1997; Cobellis *et al.* 1999; Chieffi *et al.* 2000).

Previous studies have reported detrimental effects of oestrogenic compounds on fertility at all levels of the brain-pituitary-gonad axis in teleost fish (Tsai *et al.* 2005; Filby *et al.* 2006; Zhang *et al.* 2008), including impairment of spermatogenesis (Van der Ven *et al.* 2003; Van den Belt *et al.* 2004; Pawloswki *et al.* 2004; Van der Ven *et al.* 2007), and inhibition of androgen synthesis, either directly on Leydig cells (Loomis & Thomas 2000; Govoroun *et al.* 2001; Baron *et al.* 2005) or via feedback on hypothalamus and pituitary to control synthesis and release of gonadotropins (Dickey & Swanson 1998; Kobayashi *et al.* 2001; Huggard-Nelson *et al.* 2002; Banerjee & Khan 2008). However, it is not known exactly which step(s) in the developmental sequence constituting spermatogenesis is/are affected by oestrogens in fish. This may be partially related to the fact that quantitative morphometry has not yet been applied to the evaluation of oestrogenic treatment effects on fish testis.

Our present knowledge on the role of androgens in fish spermatogenesis is mainly based on studies conducted in prepubertal individuals. In immature Japanese eel testis containing type A and a few type B spermatogonia only, androgens can induce rapid proliferation of spermatogonia and their terminal differentiation into spermatozoa under tissue culture conditions (Miura *et al.* 1991). Androgen treatment of juvenile male African catfish (*Clarias gariepinus*) induced precocious testis growth, spermatogonial proliferation and entry into meiosis

(Cavaco *et al*. 1998), and the rise of androgen plasma levels in pubertal Chinook salmon (*Oncorhynchus tshawytscha*) coincided with the start of rapid spermatogonial proliferation (Campbell *et al.* 2003). However, scarce information is available on the role of androgens in adult spermatogenesis in fish. In mammals, it is well known that the first (pubertal) wave of spermatogenesis differs from adult spermatogenesis in both regulation and timing; for example, androgen requirements differ between pubertal start and adult maintenance of spermatogenesis (Handelsman *et al.* 1999).

An oestrogen-induced decrease of testosterone levels has given valuable information on androgen-dependent stages in rodent spermatogenesis, revealing a slower conversion of round to elongated spermatids (O'Donnell *et al.* 1994). A similar approach was used in the current report to investigate the role of androgens in adult fish spermatogenesis. We studied androgen release and expression of steroidogenesis-related genes in testis of adult zebrafish (*Danio rerio*) after exposure to 17β-oestradiol (E_2) *in vivo*, and in zebrafish testicular tissue exposed to E_2 *ex vivo*. Moreover, we determined the changes in absolute weight of the different germ cell stages and we quantified germ cell proliferation and apoptosis.

Material and methods

Fish stocks

Unless otherwise stated, adult (>90 dpf) male Tübingen AB strain 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 E_2

Male zebrafish were exposed to either 0.3 nM E_2 (Sigma-Aldrich; St. Louis, MO, USA), 10 nM E_2, or control conditions (same volume of deionised water only; see below) for either 6 or 21 days during *in vivo* exposure experiments. The dose of 0.3 nM was chosen since it is close to the K_d-values of the zebrafish Esr proteins (Menuet *et al.* 2002), while the dose of 10 nM was selected based on its reported capacity to disturb adult zebrafish spermatogenesis (Van der Ven *et al.* 2003). Since 0.3 nM E_2 did not elicit any deleterious effect on spermatogenesis after 21 days of

exposure (see Suppl. Fig. S1; http://joe.endocrinology-journals.org/supplemental/), we decided to continue our studies with the 10 nM dose only. Sample sizes for the different experiments or types of analyses varied from 4 to 13 fish per treatment (see respective figure legends). A 10 µM E_2 stock solution was prepared in deionised water by extensive stirring at 40°C, which was then further diluted to 10 nM in aquarium water. Exposure was performed in glass tanks containing 18 L water maintained at 27.1 ± 0.2°C under constant aeration. Fish were transferred to the experimental tanks 24 h before initiating each exposure period. The exposure environment was refreshed every day by moving the fish to a second set of identically prepared tanks. After exposure, 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. Testis samples were used for acute *ex vivo* steroid release bioassays or processed for histological evaluation, morphometrical quantification, immunohistochemistry, western blot, or gene expression analysis (for detailed experimental procedures, see Supplemental Information; http://joe.endocrinology-journals.org/supplemental/).

Ex vivo exposure to E_2

To determine whether oestrogens are able to exert direct effects on zebrafish spermatogenesis and testicular androgen production, an *ex vivo* organ culture system for zebrafish testis was used (Leal *et al*., 2009a). In this system, the influence of interindividual variation is reduced by incubating the two testes of each fish in parallel, such that one testis served as control for the contra-lateral one. Basal culture medium was supplemented with 50 ng/ml recombinant gilthead seabream (*Sparus aurata*) insulin-like growth factor 1 (Igf1; Prospec-Tany Technogene, Rehovot, Israel), which was added to support spermatogenesis in an androgen-independent manner (Leal *et al.* 2006). This setting was chosen to study direct E_2 effects on the testicular steroidogenic system while avoiding dramatic changes in the cellular composition of the testis explant in the absence of compounds supporting spermatogenesis (Leal *et al.* 2009a). The E_2 stock solution used in these experiments was prepared as described above. Incubation took place in a humidified air atmosphere at 25ºC, and medium was refreshed every three to four days. When androgen release by testicular tissue was to be measured, the culture environment was refreshed with the same frequency by transferring the nitrocellulose membranes supporting the tissue explants to fresh culture wells (and both medium and agarose blocks were stored together at -25°C). After incubation (exposure periods are specified for each experiment performed; see below), testis

tissue explants were collected, weighed and processed for morphological evaluation or gene expression analysis (for detailed experimental procedures, see Supplemental Information; http://joe.endocrinology-journals.org/supplemental/), while both medium and agarose blocks were stored together at -25°C for quantification of 11-ketotestosterone (11-KT) levels by radioimmunoassay (Schulz *et al.* 1994). For that purpose, incubation media and agarose cylinders were transferred to a glass tube for homogenization (Ultra-turrax T25; Janke & Kunkel Ika-Labortechik, Staufen, Germany) and steroid extraction with diethyl ether (four times with 5 ml solvent each). Recovery studies using tritiated androgens showed that only the steroids in the incubation medium, but not those associated with the agar cylinder, were effectively extracted, resulting in a relatively low recovery of 47±1% (n=8). The results were corrected accordingly, and are expressed as pg 11-KT released per mg of testis tissue incubated.

In a first series of experiments, testicular tissue explants were exposed to either 10 nM E_2 or control conditions (basal medium only) for either 30 h (studies on steroid release only; n=9 explants per treatment), 6 days (studies on steroid release, morphology and gene expression; n=6-9 explants per treatment), or 12 days (studies on morphology only; n=6-9 explants per treatment). The 30 h incubation period was selected based on our recent studies showing that after 48 h under basal conditions, the activity of the steroidogenic system had decreased to less than 10% of the starting level while at 30 h the responsiveness to an acute stimulation was intact (Leal *et al.* 2009a). The 6 and 12 days incubation periods were selected in order to evaluate possible medium- to long-term effects of E_2 on zebrafish spermatogenesis.

In a second series of experiments, testicular tissue explants were exposed to 0.5 µM of the adenylate cyclase activator forskolin (prepared in DMSO; Sigma-Aldrich) alone or in the presence of 10 nM E_2 for 6 days (studies on steroid release and gene expression; n=8 explants per treatment). This experimental setting was chosen according to recent studies demonstrating the capacity of forskolin to partially prevent down-regulation of the zebrafish testicular steroidogenic system under culture conditions (Leal *et al.* 2009a), which allowed examination of possible, direct effects of E_2 on the activity of the steroidogenic system in a medium-term time frame.

Acute *ex vivo* steroid release bioassays

The steroidogenic capacity of testicular tissue after 6 days of *in vivo* oestrogen exposure was evaluated using an acute *ex vivo* steroid release bioassay previously described for African catfish (Schulz *et al.* 1994) and adapted for zebrafish testis

explants. For this experiment, adult outbred zebrafish were used. The testes of either 10 nM E_2-treated or control fish (n = 10-12 fish per group) were collected after a 6 days *in vivo* oestrogen exposure period and the two testes were immersed in D-PBS+. One testis was incubated in basal medium (the same as used for *ex vivo* exposure to E_2, but excluding retinoic acid and Igf1), serving as control for the contra-lateral testis, which was challenged with either 0.5 µM forskolin or a 1/10 dilution of single-chain, recombinant zebrafish luteinizing hormone (Lh) stock solution (preparation of recombinant zebrafish Lh is described briefly in Supplemental Information, http://joe.endocrinology-journals.org/supplemental/). Testis tissue was immersed in 200 µl medium in 96-well flat-bottom plates (Corning) in a humidified air atmosphere at 25ºC for 18 h. After incubation, testis explants were weighed and discarded, and the incubation medium was heated at 80°C for 1 h, centrifuged for 30 min (16,000 g; 4°C) and the supernatant was stored at -25°C until direct quantification (*i.e.* without extraction) of 11-KT levels by radioimmunoassay as reported (Schulz *et al.* 1994). The results were expressed as pg 11-KT released per mg of testis tissue incubated.

Statistical analysis

For the *in vivo* experiments, differences between treatments were analysed by the Student t-test with two-tailed P-value (in some cases, data were log transformed to achieve an equal variance), except for the gene expression analysis after 21 days *in vivo* E_2 exposure in which, due to differences in the number of individuals between groups, the Mann-Whitney non-parametric test was used. Comparison of groups in the acute 11-KT release assay for both *in vivo* and *ex vivo* experiments was done with one-way ANOVA followed by the Student-Newman-Keuls test. For the *ex vivo* gene expression experiments, differences between treatments were tested for statistical significance using the paired t-test (when necessary, data were log transformed to achieve an equal variance). A significance level (P) of 0.05 was applied in all the statistical analyses, which were performed using the Prism4 software package (GraphPad software, San Diego, CA).

Results

Effects of *in vivo* exposure to E_2 on zebrafish testicular physiology

In vivo exposure to oestrogen, either for 6 or 21 days, did not induce significant changes in body weight or GSI (data not shown), while the decrease in total testis

weight (from 6.21 ± 0.54 mg to 4.68 ± 0.32 mg) observed after 21 days reached statistical significance.

Qualitative morphological (Fig. 1A-C) and quantitative morphometric analysis (Fig. 1D, E) of testis tissue samples collected after both 6 and 21 days *in vivo* exposure to E_2 revealed a significant inhibitory effect on zebrafish spermatogenesis. At 6 days, the main changes in the morphometric analysis were recorded among type B spermatogonia, primary spermatocytes, and secondary spermatocytes, which decreased to 54-60% of control levels (Fig 1D). After 21 days of oestrogen exposure, the mass of type B spermatogonia, primary and secondary spermatocytes, and spermatids decreased further and significantly (*e.g.* down to ~19% of control levels in the case of spermatids), whereas the mass of type A spermatogonia increased to ~220% of control levels (Fig. 1E). Expression levels of synaptonemal complex protein 3 like (*sycp3l)* mRNA, a zebrafish homologue to a marker for primary spermatocytes (De la Fuente *et al.* 2007), correlated well with the reported decrease in the presence of primary spermatocytes in zebrafish testis after *in vivo* E_2 exposure (Fig. 1F). No differences were observed in the mass of spermatozoa or other cell types between treatment groups at both sampling times (Fig 1D, E). A very low incidence of apoptotic germ cells (strongly condensed, darkly stained nuclei in toluidine-blue-stained sections), and no difference between control and E_2-treated groups at both sampling times were observed by morphometrical analysis (Fig. 1D, E).

Searching for a mechanistic basis for the reduced mass of type B spermatogonia and spermatocytes, immunocytochemical detection of the G_2-phase cell cycle marker phosphorylated histone H3 (PH3) was used to assess proliferative activity after 6 days of E_2 exposure. As shown in Suppl. Fig. S2 (see Supplemental Information; http://joe.endocrinology-journals.org/supplemental/), proliferating (*i.e.* PH3-positive) single spermatogonia, as well as cysts containing 4 or more proliferating germ cells (spermatogonia or spermatocytes), were found in greater numbers on sections from control than from E_2-treated testes. Quantification of this observation showed that treatment with E_2 reduced the number of proliferating single germ cells as well as cysts of spermatogonia/spermatocytes significantly (to ~53% and ~37% of control levels, respectively; Fig. 1G).

We then examined the assumption that *in vivo* oestrogen exposure inhibited the testicular steroidogenic system. We quantified mRNA and protein levels of cytochrome P450 17α-hydroxylase/17-20-lyase (*cyp17a1*/Cyp17a1), a steroidogenic enzyme required for androgen production, the mRNA amounts of steroidogenic acute regulatory protein (*star*), the protein controlling the rate-limiting step in steroidogenesis (*i.e.* the transfer of cholesterol from the outer to the inner

mitochondrial membrane), as well as the mRNA amounts of both gonadotropin receptors (luteinizing hormone receptor, *lhr*, and follicle-stimulating hormone receptor, *fshr*).

Effects of *ex vivo* exposure to E_2 on zebrafish testicular physiology

Morphological evaluation of testicular tissue explants exposed to E_2 for 6 and 12 days *ex vivo* revealed no clear disruptive effect on spermatogenesis (data not shown), in contrast to the results obtained in the testes of *in vivo* E_2-exposed fish. As regards gene expression, however, *lhr* mRNA levels showed an increase (to 184% of control levels; Fig. 3A) after 6 days of *ex vivo* exposure to E_2, as observed after 6 days of *in vivo* exposure**,** while mRNA amounts of *fshr* decreased (to 65% of control levels; Fig. 3A). Also the mRNA levels of *star* and *cyp17a1* were decreased to almost half of control levels (Fig. 3A) and responded similarly as in the *in vivo* experiments, though not as prominently for *cyp17a1*. However, it should be noted that the steroidogenic system undergoes a spontaneous down-regulation under *ex vivo* culture conditions, reflected by a decrease in *cyp17a1* and *star* mRNA levels (Leal *et al.* 2009a). Hence, it seems more appropriate to state that E_2 further enhanced the spontaneous down-regulation of *star* and *cyp17a1* mRNA levels observed *ex vivo* (Fig. 3A, left panel). However, the continuous presence of 0.5 µM forskolin (Fig. 3A, right panel) reduced the additional down-regulatory effect of E_2 on *star* mRNA levels, while it abolished such an effect on *cyp17a1* mRNA levels.

After 30 h of tissue culture *ex vivo* and during the first 3 days in the continuous presence of forskolin, there was no change in the cumulative testicular release of 11-KT between groups incubated in the absence or presence of E_2 (Fig. 3B, left and middle panel). Despite the protective effect of forskolin on E_2-induced down-regulation of *star* and *cyp17a1* mRNA levels, *ex vivo* exposure to E_2 for 6 days was reflected in a significantly lower amount of 11-KT released from culture days 3 to 6 (Fig. 3B, right panel).

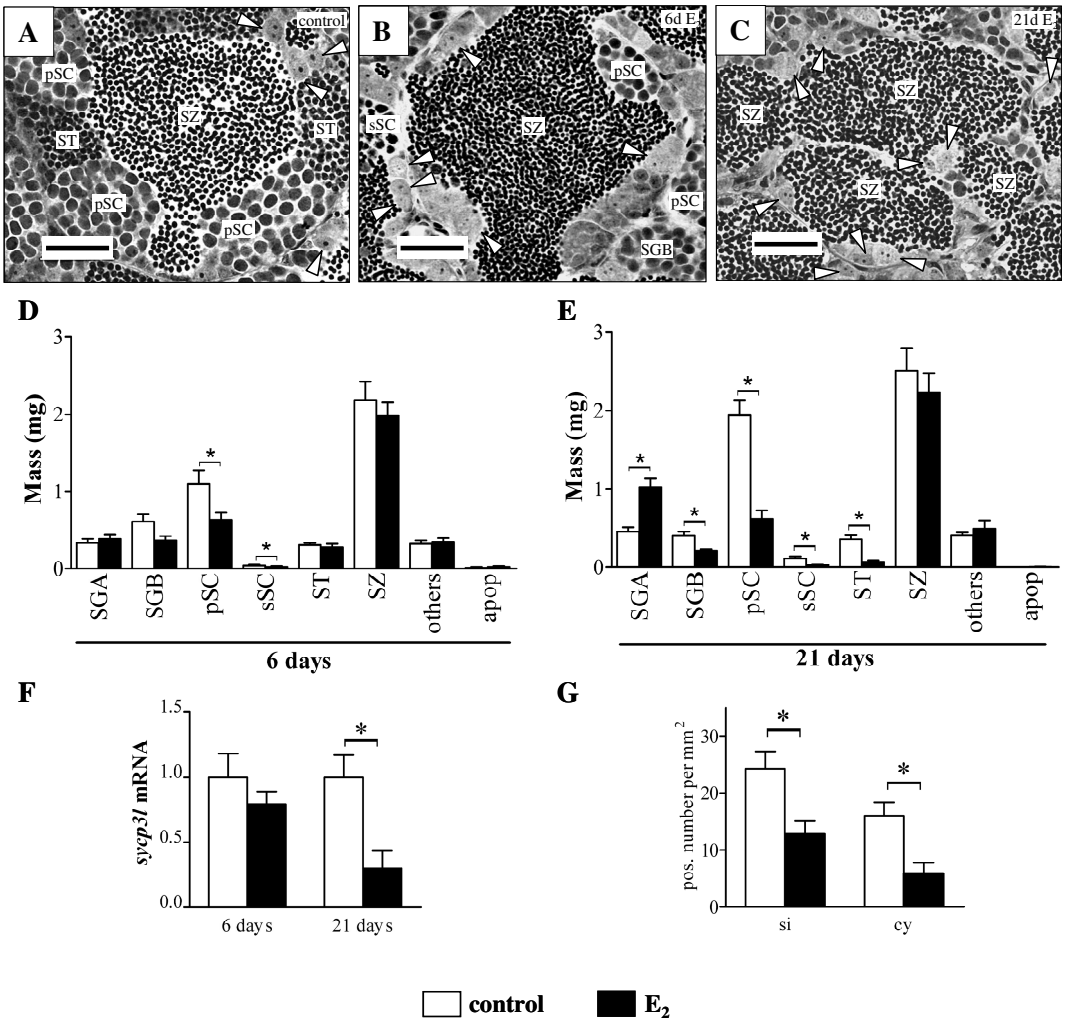
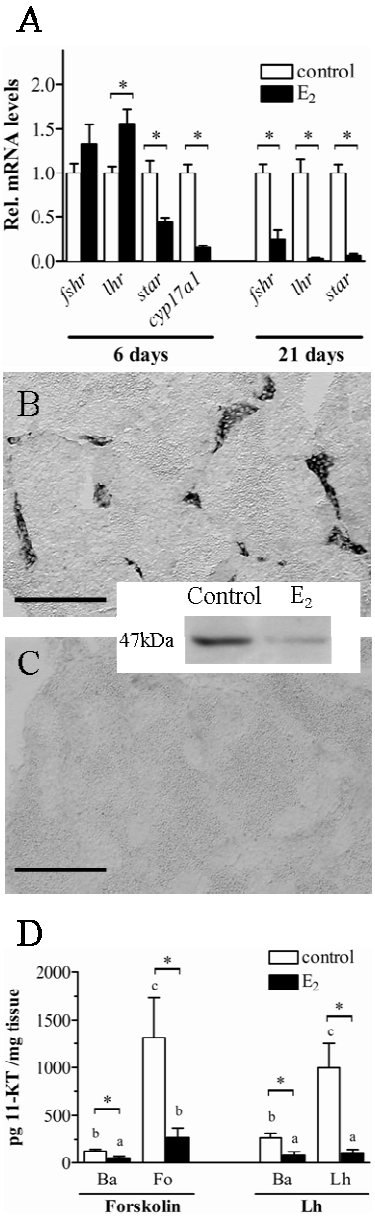


Figure 1 Zebrafish testicular physiology after exposure to 10 nM E_2 *in vivo*. Zebrafish testis sections from control fish (A), or from E_2-treated fish 6 days (B) or 21 days (C) after the start of exposure. Morphometric analysis (n=5-7 fish per group) of zebrafish testis sections, presenting data as mass (mg) of testicular cell types, 6 days (D) or 21 days (E) after the start of exposure to 10 nM E_2. (F) Relative amounts of *sycp3l* mRNA, normalised to 18S ribosomal RNA, in control and E_2-exposed males (n=6-13 fish per treatment). (G) Germ cell proliferation after 6 days of E_2 exposure (n=4 fish per treatment). Phosphohistone H3 (PH3)-positive number of single nuclei of type A spermatogonia (si) and cysts (cy) containing ≥4 PH3-positive nuclei of spermatogonia/spermatocytes per mm^2 were counted. Arrowheads and SGA, type A spermatogonia; SGB, type B spermatogonia; pSC, primary spermatocytes; sSC, secondary spermatocytes; ST, spermatids; SZ, spermatozoa; apop, apoptotic cells. Bars marked with ∗ are significantly different from their respective controls ($P<0.05$). Scale bars in (A), (B) and (C) = 25 µm.



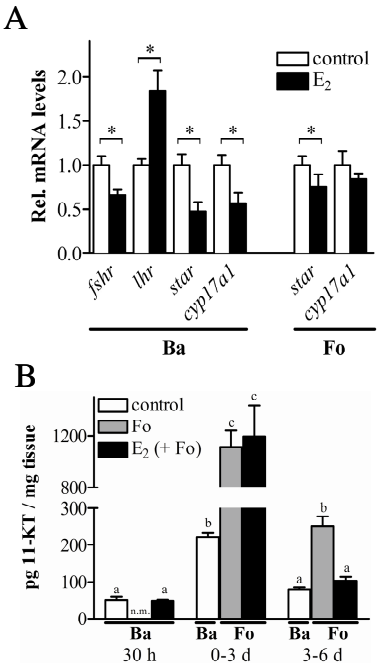


Figure 3 Steroidogenic capacity of zebrafish testis after exposure to 10 nM E_2 *ex vivo*. (A) Relative mRNA levels of *fshr*, *lhr*, *star* and *cyp17a1* after 6 days of E_2 *ex vivo* exposure either in the absence (basal; Ba) or the presence of 0.5 µM forskolin (Fo) (n=8 explants per group). Data are shown as relative values of respective transcript amounts measured in control fish. (B) Cumulative androgen release after *ex vivo* E_2 exposure for 30 hours under basal conditions and for 3 or 6 days (release was measured from 0 to 3 and from 3 to 6 days) in the absence or presence of 0.5 µM forskolin (n=8 explants per group). Androgen release is expressed as amount of 11-KT produced per unit weight of testis tissue incubated. In panel (A), bars marked with ∗ are significantly different from their respective controls, while different letters denote statistical differences in panel (B) ($P<0.05$). n.m., not measured.

Figure 2 Steroidogenic capacity of zebrafish testis after exposure to 10 nM E_2 *in vivo*. (A) Relative mRNA levels of *fshr*, *lhr*, *star* and *cyp17a1* after either 6 or 21 days of E_2 exposure (n=6-13 fish per group). Data are shown as relative values of respective transcript amounts measured in control fish. Cyp17a1 immunodetection on transversal zebrafish testis cryosections obtained from (B) control fish and (C) E_2-exposed fish. The inset between B and C shows Cyp17a1 protein amounts detected by Western blot in testicular lysates obtained from both treatment groups. (D) *Ex vivo* acute androgen release in the absence (Ba: basal) or presence of either 0.5 µM forskolin (Fo) or recombinant zebrafish Lh by zebrafish testis tissue obtained from fish exposed to E_2 *in vivo* for 6 days (n=5-6 fish per treatment). Androgen release is expressed as amount of 11-KT produced per unit weight of testis tissue incubated. Bars marked with ∗ are significantly different from their respective controls ($P<0.05$). For each compound in panel (D), different letters denote statistical differences ($P<0.05$). Scale bars in (B) and (C) = 100 µm.

Discussion

Our data demonstrate oestrogen-induced disruption of the spermatogenic process in adult zebrafish. Previous studies reported similar disruptive effects of oestrogens on the testis of sexually mature zebrafish (Van der Ven *et al.* 2003; Van den Belt *et al.* 2004; Van der Ven *et al.* 2007) and other teleost species (*e.g.* Kinnberg & Toft 2003; Pawlowski *et al.* 2004; Chaves-Pozo *et al.* 2007), but the precise stage of inhibition of spermatogenesis was not determined. Therefore, the mechanistic basis of oestrogen-induced disruption of germ cell development in adult male teleost testis has remained unclear.

Different from earlier stereological analyses on testes of E_2-exposed zebrafish (Van der Ven *et al.* 2003; Christianson-Heiska *et al.* 2004) or guppy (*Poecilia reticulata*; Nielsen & Baatrup 2006), a distinction was made in the spermatogonial compartment between early (type A) and late (type B) spermatogonia in the current study (see Leal *et al.* 2009b, for a comprehensive description of the spermatogonial generations in zebrafish). Implementation of this distinction, quantification of the absolute masses of the different germ cell types, and analysis of germ cell proliferation and apoptosis enabled us to pinpoint the stages of spermatogenesis affected by the oestrogen treatment. Thus, E_2 exposure resulted in two main effects: (i) the reduction, but not the abolishment, of proliferation of type A and type B spermatogonia as demonstrated by quantifying PH3-positive germ cells, and (ii) the blockade of differentiation of type A into type B spermatogonia, as demonstrated by the accumulation (*i.e.* increased mass) of type A spermatogonia. Jointly, these effects resulted in a depletion of developmental stages beyond type A spermatogonia, as demonstrated by the significant decrease in the masses of type B spermatogonia, spermatocytes, and spermatids. Germ cell depletion was increasingly prominent with their progressive differentiation (reductions to 53, 31, 31, and 19% of control values for type B spermatogonia, primary, secondary spermatocytes, and spermatids, respectively). This may indicate that oestrogen-mediated inhibition of spermatogenesis is based on (at least) two components, the reduced availability of type B spermatogonia, as demonstrated in the present study, and inhibition of germ cell entry into, or progress through, meiosis and spermiogenesis. To clarify the second component, however, other experiments will be required. In any case, the absence of a clear inhibitory effect of oestrogen on meiosis/spermiogenesis *ex vivo* renders it unlikely that oestrogen affects these stages directly, while the presence of meiotic and spermiogenic stages shows that meiosis and spermiogenesis are compatible with elevated oestrogen concentrations in zebrafish.

Our results on oestrogen-mediated reduction of germ cell proliferation are in accordance with previous observations in fish and amphibian species (Song & Gutzeit 2003; Tsai *et al.* 2005; Chaves-Pozo *et al.* 2007), although these studies did not determine the changes in germ cell masses (*i.e.* provided data on relative changes in the germ cell compartment), and did not always identify the specific stage of spermatogenesis affected. Interestingly, Miura *et al.* (2003) reported an E_2–triggered molecular mechanism, which involves up-regulation of platelet-derived endothelial cell growth factor, and subsequently stimulates self-renewal divisions of spermatogonial stem cells, the first generation of type A spermatogonia, in immature Japanese eel testis *ex vivo*. Although we found an increased mass of type A spermatogonia, this was associated with a reduced proliferation activity of these cells. In conjunction with no change in the low rate of apoptosis, this can be explained by a block of differentiation, and ensuing accumulation, of type A spermatogonia. Therefore, juvenile eel may respond differently to oestrogens than adult zebrafish (see also below for a discussion on oestrogen feedback effects on the brain/pituitary level). Moreover, in eel testis *ex vivo,* the stimulatory effect decreased with increasing (0.01 – 1 ng/ml) oestrogen doses (Miura *et al.* 1999), the concentration used in the present study being ~3 times higher than the maximum concentration used for eel testis.

Different from previous reports, where an increased incidence of germ cell apoptosis was proposed as an important cause for oestrogen-induced impairment of spermatogenesis in other fish species (Weber *et al.* 2004; Chaves-Pozo *et al.* 2007), our morphometric analysis could not confirm this observation for zebrafish.

Based on the data available from mammalian models (Bartke *et al.* 1977; Akingbemi *et al.* 2003; Gould *et al.* 2007), we hypothesised that oestrogen treatment inhibits androgen synthesis, thereby indirectly affecting spermatogenesis. Indeed, exposure of adult zebrafish to E_2 *in vivo* suppressed the testicular steroidogenic capacity, as reflected by down-regulation of key steroidogenesis-related genes (*cyp17a1* and *star*) and the lower acute testicular 11-KT release (under basal conditions). Moreover, the E_2 treatment suppressed the androgen release response to an acute gonadotropic stimulation with biologically active recombinant zebrafish Lh even though *lhr* mRNA levels were elevated and *fshr* mRNA showed no significant changes after oestrogen treatment. These seemingly contradictory observations, which are reported for the first time in any fish species but have been previously reported in oestrogen-exposed mice (Fukuzawa *et al.* 2004), can be reconciled assuming that oestrogen exposure interfered with the signalling pathways activated by Lh, but downstream of its receptor, in steroidogenic cells. Since also forskolin, an activator of the adenylate cyclase, induced a much weaker

response in tissue from E_2-treated than from control fish, we speculate that oestrogens may target one or more components of the $G\alpha_S$ – adenylate cyclase – PKA pathways. Therefore, we conclude that oestrogenic treatment induced a state of androgen-insufficiency in zebrafish by disturbing the testicular steroidogenic system at different levels, as previously suggested for other teleost fish species (Sohn *et al.* 1998; Loomis & Thomas 2000; Andersen *et al.* 2006; Filby *et al.* 2006; Chaves-Pozo *et al.* 2007; Meier *et al.* 2007; Arukwe 2008; Blum *et al.* 2008; Juskosky *et al.* 2008; Zhang *et al.* 2008).

In search of a mechanism explaining the oestrogen-induced disruption at the testicular level, we thereafter studied possible direct effects of E_2 on testis physiology using a recently developed tissue culture system for zebrafish testis explants (Leal *et al.* 2009a). After 6 days *ex vivo* oestrogen treatment, zebrafish testis showed significantly reduced *star* and *cyp17a1* mRNA levels as compared with control levels suggesting a direct inhibition of E_2 on testicular steroidogenic system. However, it should be mentioned that the expression of steroidogenic enzymes, and thus the steroidogenic capacity, shows a spontaneous down-regulation under prolonged *ex vivo* culture conditions (Baron *et al.* 2005; Leal *et al.* 2009a). Hence, we further studied the *ex vivo* effects of the E_2 exposure on the steroidogenic potential of zebrafish testis (i) within the first 30 hours of culture, when zebrafish testicular steroidogenic system is still responsive to short-term acute stimulations and thus can be considered as relatively intact (Leal *et al.* 2009a), and (ii) during 6 days of culture in the presence of forskolin, which partially prevents the aforementioned down-regulation of the system under culture conditions, probably by up-regulating the expression of several steroidogenesis-relevant genes through the cAMP/PKA pathway (Schwartz & Roy, 2000; Manna *et al.* 2003; Leal *et al.* 2009a). Under these culture conditions, oestrogen exposure was only able to decrease androgen release from day 3 to 6 possibly in association with the slight, but significant, down-regulation of *star* mRNA transcription observed at the end of the incubation period. These results suggest that the direct effects exerted by oestrogens in the zebrafish testicular steroidogenic system may be relatively minor under the conditions tested in the current study, since E_2 exposure could neither decrease androgen output when the system was still intact at 30 hrs of culture nor at 3 days of treatment. Previous studies in other fish and amphibian species, however, have reported significant inhibitory effects of oestrogenic compounds on both testicular androgen release and steroidogenic enzymes expression in tissue culture exhibiting, therefore, direct suppressive actions of oestrogens on testicular androgenesis (Pierantoni *et al.* 1986; Loomis & Thomas 2000; Baron *et al.* 2005). Notably, such effects were only evident at very high

oestrogen concentrations (>367 nM), making these results difficult to interpret in context with physiological oestrogen concentrations or to compare with our observations in the present study using 10 nM E_2. In Atlantic croaker (*Micropogonias undulutatus*), direct suppression of testicular androgen production by very high oestrogen concentrations (>36.7 μM) was shown to be rapid (within 5 min) and was transduced by a membrane-associated ER (Loomis & Thomas 2000). Recently, a membrane associated ER, homologous to mammalian *GPER*, has been cloned from zebrafish, and is expressed in testis (Liu *et al.* 2009). The E_2 concentration used in the current study (10 nM) could have been sufficient to activate the zebrafish Gper. However, the direct effects of oestrogen in the zebrafish testis were comparatively weak and possibly mediated via a nuclear Er, considering the prolonged time required for them to become evident, while the binding of E_2 to zebrafish Gper, although of high affinity (K_d=2.8nM), was characterised by both rapid association and dissociation (Liu *et al.* 2009).

In contrast to the results obtained in the testes of E_2-exposed zebrafish *in vivo*, morphological and/or morphometrical evaluation of testis tissue exposed to E_2 *ex vivo* revealed no clear disruption of spermatogenesis which together with the minor direct effect exerted by oestrogen on the steroidogenic system, suggests that the E_2-induced inhibitory effects on zebrafish testis functions mainly involve feedback mechanisms on the hypothalamus-pituitary system. Sex steroid, either androgens or oestrogens, feedback on gonadotropin synthesis and release is well established in fish (Dickey & Swanson 1998; Kobayashi *et al.* 2001; Huggard-Nelson *et al.* 2002; Banerjee & Khan 2008). In zebrafish, information on oestrogen effects on gonadotropin levels (either subunits mRNA expression levels or plasma levels) is scarce. Recently, Lin & Ge (2009) described direct oestrogenic stimulation of *fshb* and *lhb* transcript levels in primary zebrafish pituitary cell culture, similar to previous findings in the closely related goldfish (*Carassius auratus*; Huggard-Nelson *et al.* 2002). While no information is available on *in vivo* oestrogen feedback effects on gonadotropin subunit mRNA levels in zebrafish, respective data (as well as gonadotropin plasma amounts) are available in goldfish. For this species, oestrogen treatment abolished the up-regulation of both pituitary *fshb* mRNA levels and Lh plasma levels observed in ovariectomized individuals (Kobayashi *et al.* 2001). Furthermore, treatment of sexually mature goldfish with an oestrogen receptor antagonist increased circulating Lh levels (Billard & Peter 1977). Finally, pre-treatment of late recrudescent goldfish of mixed sex with E_2 suppressed GnRH-mediated elevation of *fshb* and *lhb* expression both *in vivo* and *in vitro* (Huggard-Nelson *et al.* 2002). The latter has been reported recently in medaka (*Oryzias latipes*; Zhang *et al.* 2008) as well. These data indicate that E_2 inhibited the

Gnrh/Gnrh-receptor signalling system, and that these effects apparently overrule the direct, stimulatory effects of oestrogens on gonadotropin subunit expression. Also signalling pathways towards GnRH neurons, like γ-aminobutyric acid (GABA; Kah et al. 1992), or the recently described Kiss1/Gpr54 system (Elizur, 2009) may be influenced by estrogens in teleost fish. Therefore, E_2-induced gonadotropin insufficiency seems the main factor contributing to the observed down-regulation of androgen production in the zebrafish testis, which in turn results in an interruption of spermatogenesis.

Androgen depletion or blocking of androgen signalling had a marked effect on meiotic and postmeiotic events of rodent spermatogenesis, whereas effects on spermatogonial proliferation were limited (O'Donnell *et al.* 1994; França *et al.* 1998; De Gendt *et al.* 2004). This differs from the situation reported in different fish species. For instance, in juvenile testes containing only spermatogonia, androgen treatment stimulated rapid spermatogonial proliferation both in tissue culture experiments (Miura *et al.* 1991) and *in vivo* (Cavaco *et al.* 1998). However, it was not known if, after the first (pubertal) wave of spermatogenesis, also spermatogonial proliferation during adult spermatogenesis depends on androgens. In mice, for example, more androgen is required for the first than for subsequent waves of spermatogenesis (Handelsman *et al.* 1999). The present study shows that oestrogen-induced androgen insufficiency reduced proliferation of type A spermatogonia as well as their differentiation into type B spermatogonia in adult zebrafish testis, and strongly reduced the number of meiotic and haploid germ cells produced. To our knowledge, this is the first study localising specific, androgen-dependent germ cell stages during adult spermatogenesis in teleost fish. This observation correlates well with both the high level of *ar* mRNA expression in Sertoli cells contacting type A spermatogonia in zebrafish testis (De Waal *et al.* 2008) and the elevation of circulating androgen levels in Chinook salmon at the time when seasonal spermatogonial proliferation starts (Campbell *et al.* 2003).

Declaration of interest

The authors declare there is no conflict of interest that could have prejudiced the impartiality of the research reported.

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Supplemental information

Supplementary material & methods

Preparation recombinant zebrafish Lh

Recombinant zebrafish Lh was produced as a single-chain molecule by joining the Lh β subunit with its α subunit coding sequences using a Gly/Ser linker (GGGSGGGSGGGSGGG) coding sequence, ligated into a mammalian expression vector containing an N-terminal TEV-cleavable His tag (U-Protein Express BV, Utrecht, The Netherlands). The resulting plasmid was used for transient transfection of 1 L HEK293-EBNA1 suspension cultures growing in Freestyle medium (0.02% v/v FCS; Invitrogen) (Marx *et al.* 2008). Four hours post transfection 0.9% v/v Primatone (Kerry Biosciences, Naarden, The Netherlands) was added. Five days post transfection the medium was harvested by centrifugation, concentrated, and dialyzed into 20 mM Tris (pH 8.0), 500 mM NaCl using a Quickstand hollow fiber (GE Healthcare, Diegem, Belgium) with a 10 kDa molecular weight cut-off cartridge (GE Healthcare).The protein solution was then loaded onto a 1 ml HisTrap column (GE Healthcare) and eluted with a gradient of imidazole (Merck, Darmstadt, Germany). Peak fractions were analyzed using SDS PAGE and Western blotting (described below), and relevant fractions were pooled and dialyzed into 20 mM Tris (pH 7.5), 500 mM NaCl and loaded on a 2 ml Concanavalin-A Sepharose column (GE Healthcare). Elution was performed using a gradient of □-D-methyl mannopyranoside (Sigma-Aldrich). Peak fractions were analyzed by Western blotting, pooled, concentrated and loaded on a HiLoad 16/60 Superdex 200 pg column (GE Healthcare).

After analysis, relevant fractions were pooled, dialyzed into PBS and concentrated for use. In pilot studies, different dilutions with basal medium (1/4 – 1/25) were tested for the steroid release capacity of this preparation (data not shown). A 1/10 dilution was found to half-maximally stimulate androgen release and was used for further studies.

Western blot

To verify expression of recombinant zebrafish Lh, Western blotting procedures with a commercially available His tag-specific monoclonal antibody (Novagen) were conducted, following the instructions of the manufacturer.

For Cyp17a1 immunodetection, testes were homogenised in PBS (pH 7.4). After centrifugation of the homogenates (10,000 g, 15 min, 4°C), the S9 fraction was collected and the protein concentration was determined using a Bradford assay, with bovine serum albumin as standard. For the western-blot analysis, 20 µg of S9 protein was electrophoresed using a SDS-PAGE system (Mini-Protean II; Bio-Rad, Marnes-la-Coquette, France) on a 10% w/v acrylamide/bisacrylamide gel and Tris-glycine buffer (pH 8.3) as the running buffer. Pre-stained molecular weight markers (BioRad) were loaded on the gels that were either stained using Coomassie brilliant blue or blotted onto nitrocellulose membranes. Cyp17a1 protein was detected using a zebrafish Cyp17a1 antibody (Brion *et al.* in preparation) as primary antibody and goat anti-rabbit horseradish peroxidase-conjugated antibody as secondary antibody (BioRad). Visualization was achieved with an enhanced chemiluminescence detection kit (ECL; GE Healthcare).

Immunohistochemistry

Testis tissue samples from adult outbred fish were fixed overnight in 4% w/v paraformaldehyde in PBS (pH 7.4) at 4°C supplemented with 0.2% v/v picric acid (only for Cyp17a1 detection). For the detection of the phosphorylated form of histone H3 (PH3), a G2-phase marker present in cells about to proliferate, samples were dehydrated and embedded in paraffin and cut into 5 µm thick sections, according to conventional procedures. For Cyp17a1 detection, fixed tissue samples were briefly rinsed in PBS, cryoprotected by immersion in 25% sucrose for 16 h, frozen, and then sectioned on a cryostat at 12 µm thickness.

Immunodetection of PH3 was carried out exactly as described previously (Leal *et al.* 2008). Immunodetection of Cyp17a1 was performed as described previously (Menuet *et al.* 2005) with the following modifications. Sections were incubated with a zebrafish Cyp17a1 antibody (Brion *et al.* in preparation) overnight at a dilution of 1:300, the secondary antibody used was goat anti-rabbit conjugated to horseradish peroxidase (1:200). The specificity of the staining was controlled by processing adjacent sections without primary antibody or with the pre-immune serum.

After visual inspection by two different observers, only PH3-stained sections required quantification (see results sections). For that purpose, the number of single immuno-positive, large and round or slightly oval nuclei (i.e. type A spermatogonia), and the number of cysts containing ≥4 positive nuclei of spermatogonia or spermatocytes were counted. Three different fields in each of two independent sections (at least 20 µm apart from each other) were analysed per fish (n=4 fish per treatment). After measuring the tissue area of each section using image analysis, the number of positive cells or cysts per mm^2 was calculated.

Gene expression analysis

Total RNA was extracted from testis samples using either the FastRNA Pro Green Kit (*in vivo* exposure samples; MP Biomedicals, Solon, OH, USA), the RNAqueous-Micro Kit (*ex vivo* exposure samples; Ambion, Austin, TX, USA), following the manufacturers' instructions. Further processing to estimate the relative mRNA expression levels of a number of selected genes (see below) by real-time, quantitative PCR was performed as described previously (De Waal *et al.* 2008). A TaqMan Gene Expression Assay was acquired to detect the endogenous control RNA, eukaryotic 18S ribosomal RNA (TaqMan Gene Expression Assays [18S] mRNA; Applied Biosystems, Foster City, CA, USA]). Specific primers were designed to detect *cyp17a1* (forward, 5'-GGGAGGCCACGGACTGTTA-3', reverse, 5'-CCATGTGGAACTGTAGTCAGCAA-3'), *fshr* (forward, 5'-GAGGATTCCCAGTAATGCTTTCCT-3', reverse: 5'-TCTATCTCACGAATCCCGTTCTTC-3'), *lhr* (forward, 5'-CGCTCAGTACCATCCAATGCT-3', reverse, 5'-TTGAAGGCATGGCTCTCTCTATTTCT-3'), *star* (forward, 5'-CCTGGAATGCCTGAGCAGAA-3', reverse, 5'-ATCTGCACTTGGTCGCATGAC-3'), and *sycp3l* (forward, 5'-AGAAGCTGACCCAAGATCATTCC-3', reverse, 5'-AGCTTCAGTTGCTGGCGAAA-3') in a SYBR Green-based assay (Applied Biosystems).

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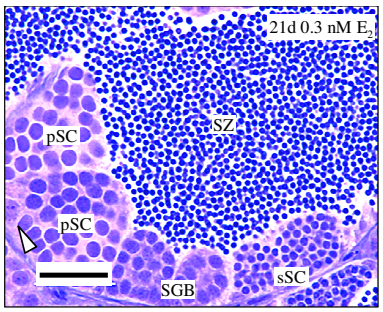
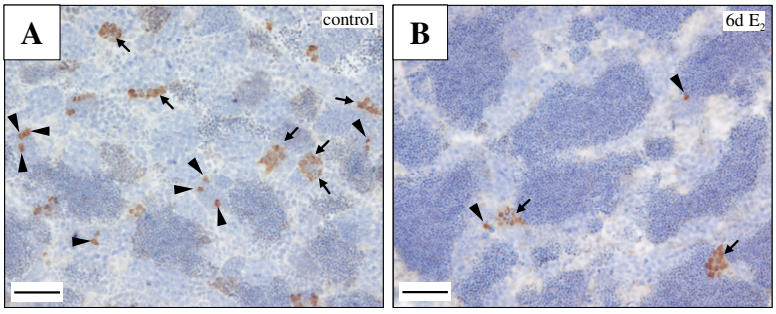
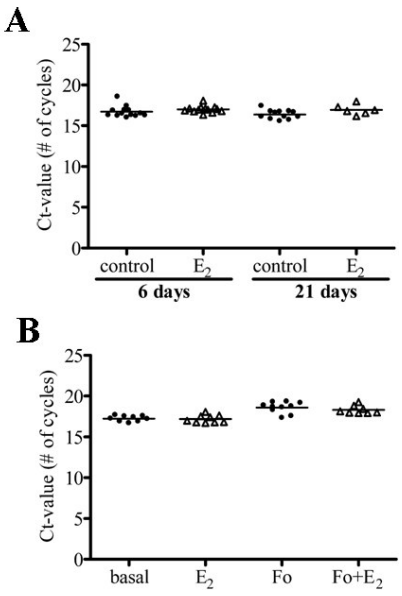


Supplemental Figure S1: Zebrafish testis section of a fish treated with 0.3 nM for 21 days after the start of exposure showing undisturbed spermatogenesis. Arrowhead, type A spermatogonia; SGB, type B spermatogonia; pSC, primary spermatocytes; sSC, secondary spermatocytes; SZ, spermatozoa. Scale bar = 25 µm.



Supplemental Figure S2: Immunodetection of the proliferation marker PH3 in representative zebrafish testis sections obtained from (A) control fish and (B) fish exposed to 10 nM E_2 for 6 days. Sections were counterstained with Mayer heamatoxilin. Arrowheads indicate proliferating single, type A spermatogonia, arrows indicate proliferating cysts of four or more spermatogonia/spermatocytes. Scale bar = 50 µm.



Supplemental Figure S3: Stability of 18S rRNA as a housekeeping gene throughout time and treatment of *in vivo* (A) and *ex vivo* (B) exposure experiments. Each circle (basal, control or forskolin, Fo) or triangle (E_2, or Fo + E_2,) in the scatter plots represents the average Ct-value of duplicate measurements for each fish or explant. No significant differences in testicular 18S rRNA Ct-values were found between control and E_2-treated fish (A), nor between respective control and E_2-treated explants (B).

5

Paracrine Effects of Insulin-like Growth Factors on Adult Zebrafish Testis

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MANUSCRIPT IN PREPARATION

Abstract

Insulin-like growth factors (IGFs) stimulate spermatogenesis across vertebrates. In adult piscine spermatogenesis, however, many aspects of IGF activity in the testis have remained elusive. Analysing germ cell-depleted mutant zebrafish revealed that the mRNAs for IGF peptides and type 1 IGF receptors are preferentially expressed in somatic cells of the testis. We demonstrated by *in situ* hybridization that the teleost- and gonad-specific *igf3* mRNA is expressed in zebrafish Sertoli cells, and that the *igf3* mRNA levels decreased in testis tissue *ex vivo*, *i.e.* deprived of gonadotropic stimulation. These levels could be restored by Fsh, also when the production of biologically active steroids was inhibited, while the weaker Lh-induced increase in *igf3* mRNA levels was abolished when androgen production was inhibited. *In vivo*, the *igf3* mRNA levels decreased in zebrafish suffering from oestrogen-induced androgen insufficiency, or increased in gonadotropin-treated zebrafish, in particular in response to Fsh. Recombinant piscine Igf1 stimulated the proliferation and differentiation of type B spermatogonia in zebrafish testis tissue culture, and we showed that this stimulatory effect was not mediated by androgens. Moreover, an additive stimulatory effect was observed in androgen/Igf1 co-incubation experiments. In conclusion, endocrine control exerted by Fsh and androgens on *igf3* mRNA expression, and the described Igf1 bioactivity suggest an important stimulatory role for paracrine IGF action on the proliferation of spermatogonia in adult zebrafish testis.

Introduction

The insulin-like growth factor (IGF) family, consisting of IGF1 and IGF2 in higher vertebrates, is a group of small single-chain polypeptides regulating different activities such as growth, proliferation, differentiation, survival and migration. These biological activities are mediated by cell surface, tyrosine kinase receptor belonging to the insulin receptor super-family, the type 1 IGF receptor (IGF1R).

IGFs exert crucial functions in male reproduction across vertebrates (Spiteri-Grech and Nieschlag 1992; Le Roy *et al*. 1999; Chandrashekar *et al*. 2004). Well established is the influence of pituitary-secreted growth hormone (GH) as a stimulator of IGF production in various tissues, such as testis, causing endocrine, paracrine and autocrine activities (Le Roith *et al*. 2001). While GH-deficient mouse models are still (albeit less) fertile (Chandrashekar *et al*. 2004), IGF1- or IGF1R-null mice are infertile (Baker *et al*. 1996), suggesting the existence of a GH-independent

regulation of testicular paracrine/autocrine IGF activities. Indeed, regulation is also exerted by gonadotropic control of the pituitary gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) on IGF1 secretion (Cailleau *et al*. 1990; Naville *et al*. 1990).

In fish, information on the regulation and the bioactivity of testicular paracrine/autocrine IGF action on adult spermatogenesis is more limited (Le Gac *et al*. 1993; 1996; Loir and Le Gac 1994; Loir 1999) and more insight into the mechanism(s) of IGF action is required. In this regard, two points require attention. First, in fish the complexity of the IGF signalling system is broader than in higher vertebrates, possibly as a result of an additional round of genome duplication (Nakatani *et al*. 2007), which resulted in two distinct IGF2 genes (*i.e. igf2a* and *igf2b*) (Wang *et al*. 2008) and two IGF1R genes (*i.e. igf1ra* and *igf1rb*) in zebrafish ((Maures *et al*. 2002), each with a differential expression pattern and biological activity during development (Maures *et al*. 2002; Schlueter *et al*. 2007; Sang *et al*. 2008). Recently, yet another IGF gene (*i.e. igf3*) has been discovered in zebrafish, medaka and Nile tilapia, teleost species from different taxonomic orders (Cypriniformes, Beloniformes, Perciformes, respectively), which was demonstrated to be distinct from known mammalian and amphibian IGF sequences and to be expressed specifically in gonadal tissue (Wang *et al*. 2008). Second, gonadotrophic control of testicular physiology differs between teleosts and higher vertebrates. In contrast to the situation in higher vertebrates, both gonadotropins act directly on piscine (*e.g.* zebrafish) Leydig cells, which express protein and mRNA of both gonadotropin receptors (Ohta *et al*. 2007; García-López *et al*. 2009, in preparation) explaining the strong steroidogenic activity reported for piscine Fsh proteins (Planas and Swanson 1995; Weltzien *et al*. 2003; Kamei *et al*. 2003; Zmora *et al*. 2007; Aizen *et al*. 2007; Kazeto *et al*. 2008; García-López *et al*. 2009, in preparation).

To further our understanding of IGF action on spermatogenesis in adult fish, zebrafish were chosen as a well-established model to study reproductive physiology. The present study set out to elucidate three main aspects of IGF activity in zebrafish testis: (i) mRNA expression patterns of IGF peptides and receptors, (ii) effects of recombinant zebrafish gonadotropins (De Waal *et al*. 2009) and sex steroids on the mRNA expression of IGF peptides *in vivo* and *ex vivo*, and (iii) bioactivity of recombinant piscine Igf1 on zebrafish adult spermatogenesis *ex vivo*.

Material & Methods

Fish stocks

Sexually mature adult (>90 dpf), male wild type Tübingen AB strain zebrafish, homozygous *piwil1* (formerly known as *ziwi*) mutant [*piwil1*$^{(-/-)}$] zebrafish (Houwing *et al*. 2007) or outbred zebrafish were used in the experiments reported 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; the Life Science Faculties Committee for Animal Care and Use in Utrecht (The Netherlands), approved the experimental protocols.

Expression analysis of IGF signaling molecules in zebrafish testis

The following approaches were employed to analyze the testicular mRNA levels for the zebrafish IGF peptides (*i.e. igf1*, *igf2a*, *igf2b* and *igf3*), and type 1 IGF receptors (*i.e. igf1ra* and *igf1rb*).

First, relative mRNA levels for the zebrafish IGFs and type 1 IGF receptors were compared between testes after dissection immediately processed for real-time, quantitative PCR analysis (see Table 1 for primers used) (*in vivo*; n=28-29 control fish from several exposure experiments), and testes cultured *ex vivo* under basal conditions for 7 days (n=38 testis explants from several exposure experiments), as described previously (Leal *et al*. 2009b). In zebrafish testes cultured *ex vivo* under basal conditions, the steroidogenic system and its response to appropriate stimuli is spontaneously and rapidly down-regulated (Leal *et al*. 2009b). This allowed investigating expression of possible hormonally modulated factors, influencing the spermatogenic process. For these purposes, Tübingen AB strain and outbred zebrafish were used.

Second, to determine which IGF signaling molecules were expressed in the somatic testicular compartment, relative IGF and type 1 IGF receptor mRNA levels were compared between germ cell-free *piwil1*$^{(-/-)}$ (a.k.a. *ziwi*$^{(-/-)}$; Houwing *et al*. 2007) and wild type and zebrafish (n=3 fish per genetic background).

Third, to visualize the cellular expression sites of *igf3* mRNA in zebrafish testis, the messenger was detected by whole mount *in situ* hybridisation, followed by embedding of the tissue for plastic sectioning. To this end, we used testis tissue showing elevated *igf3* mRNA levels, namely testicular explants incubated in the presence of 400 nM 11-ketotestosterone (11-KT; a physiologically prominent teleost androgen in fish; Borg 1994) for 7 days (see below). To obtain a zebrafish *igf3*-specific DNA template for the synthesis of digoxigenin-labelled cRNA probe synthesis, a ~572 bp PCR product was generated with gene-specific primers 2879 and 3003 (see Table 1), as described previously (Vischer *et al*. 2003). Whole mount *in situ* hybridization was performed on testicular tissue fixed in 4% paraformaldehyde in phosphate buffered saline (PBS; pH 7.4) according to

previously described methods (Westerfield 2000; http://zfin.org/zf_info/zfbook/chapt9/9.82.html) with the modifications that tissue was treated with proteinase K (20 µg/ml; Sigma-Aldrich; St. Louis, MO, USA) at 37°C for 20 min, and that after post-fixation and before pre-hybridization, an acetic anhydride (0.25% in 0.1M triethanolamine [pH 8.0]; Merck; Darmstadt, Germany) treatment was included to reduce background. After termination of nitro blue tetrazolium/5-bromo-4-chloro-3'-indolyphosphate (both Sigma-Aldrich) staining with 3 consecutive PBS washings, tissue was fixed in 4% paraformaldehyde in PBS, dehydrated and embedded in Technovit 8100 (Heraeus Kulzer, Wehrheim, Germany) for plastic sectioning. Sections of 7 µm thickness were counterstained with 0.1% Nuclear Fast Red (in 5% aluminiumsulphate solution) for 2 min and washed in running tap water for 5 min. After a rinse in deionized water, air-dried sections were mounted with Pertex.

Hormonal modulation of zebrafish testicular IGF mRNA levels *in vivo*

In order to investigate hormone-induced changes of IGF mRNA levels *in vivo*, zebrafish were subjected to two treatments.

First, to generate androgen insufficiency *in vivo*, male Tübingen AB strain zebrafish were exposed to either 10 nM 17β-oestradiol (E_2; Sigma-Aldrich) or control conditions (deionized water only) for 6 days (n=12 fish per treatment), as described previously (De Waal *et al*. 2009); this treatment induced a strong decrease in the expression of genes involved in testicular androgen production, resulting in an inhibition of the progression of spermatogenesis.

Second, gonadotropic modulation of IGF mRNA levels was investigated by injecting male outbred zebrafish with recombinant zebrafish gonadotropins, produced as described previously (De Waal *et al*. 2009). Fish received an intra-peritoneal injection of 100 ng/g body weight of either recombinant zebrafish Fsh or Lh dissolved in PBS (volume injected 5-6 µl; n=6-8 fish per treatment); control fish received PBS only. Two hours after injection, fish were euthanized, and testes were collected for gene expression analysis. Both Fsh and Lh increased 11-KT plasma levels up to 4-fold (García-López *et al*. in preparation).

Hormonal modulation of zebrafish testicular IGF mRNA levels *ex vivo*

A recently developed organ culture system for zebrafish testis explants (Leal *et al*. 2009b) was used to assess the modulation of IGF mRNA levels, androgen release or germ cell proliferation in the following experiments:

Testicular explants (n=4-7 per concentration) from Tübingen AB strain zebrafish were exposed for 7 days to increasing concentrations (50, 100, 200, 400

nM) of 11-KT (final concentration of ethanol in the medium was 0.25%). In additional experiments to investigate germ cell proliferation (see below) testicular explants (n=7 per treatment) of outbred animals were incubated with a high dose of 11-KT (400 nM).

Single doses of recombinant zebrafish Fsh (100 ng/ml) or Lh (500 ng/ml) were used that elicited an equally strong androgen release response (García-López *et al*. in preparation). In addition, to evaluate the contribution of Fsh- and Lh-stimulated androgen synthesis on the modulation of IGF gene expression, 25 µg/ml of the 3β-hydroxysteroid dehydrogense (3βHSD) inhibitor trilostane (Chemos, Regenstauf, Germany; dissolved in ultrapure DMSO, Sigma-Aldrich) was included in some groups in the incubation media. Pilot studies showed that, at 25 µg/ml, trilostane suppressed at least 90% of the gonadotropin-induced release of biologically active 3-keto, Δ5-androgens (results not shown). Testicular explants (n=6-8 per treatment) from outbred animals were treated for 2 days.

The two testes from each fish (n=4-8 per treatment) were dissected, processed and placed in the organ culture system such that one testis served as basal solvent control (with the appropriate amounts of ethanol, PBS and/or ultrapure DMSO) for the contra-lateral one incubated with a test substance (Leal *et al*. 2009b). In the case of explants incubated with recombinant zebrafish gonadotropin plus trilostane (and their respective controls), a pre-incubation period of one hour with trilostane only was included.

All incubations took place in a humidified air atmosphere at 25ºC, and medium was refreshed every three to four days. For analysis of germ cell proliferation, the medium was refreshed with medium containing 50 µg/ml 5-bromo-2-deoxyuridine (BrdU; Sigma-Aldrich) during the last 24 h of culture. Testis tissue samples were collected for BrdU immunodetection, gene expression analysis or whole mount *in situ* hybridisation.

Igf1 effects on basal or androgen-stimulated zebrafish testis physiology

To investigate the effects of Igf1 signaling on zebrafish testis physiology (*i.e.* spermatogenesis and steroidogenesis), we exposed testis explants, utilizing the above-mentioned organ culture technique (Leal *et al*. 2009b), to recombinant gilthead seabream (*Sparus aurata*) Igf1 (Prospec-Tany Technogene, Rehovot, Israel). The choice to study the effects of Igf1 on zebrafish testis was based on papers that mammalian IGF1 stimulates germ cell proliferation *in vitro* in anamniotes (Loir and Le Gac 1994; Nakayama *et al*. 1999) or is required as a permissive factor to other stimulatory substances, whereas IGF2 has only limited effects (Nader *et al*. 1999).

Fifteen Tübingen AB strain zebrafish were used; one testis was incubated under basal conditions, while the contra-lateral was treated with one of three doses of Igf1 (12.5, 25 or 50 ng/ml). Incubation took place in a humidified air atmosphere at 25ºC for 7 days, and medium was refreshed once after four days. Testis tissue samples were collected for histological and morphometrical analysis and 5-6 explants per dose were analyzed.

The steroidogenic capacity of testicular tissue was evaluated after overnight (16 h), after the first half (0-3.5 d) or after the second half (3.5-7 d) of long term *ex vivo* exposure to a single dose of 50 ng/ml Igf1. Short term effects were evaluated, using testes from 11 zebrafish divided over two independent experiments, in an acute *ex vivo* steroid release bioassay, previously described for African catfish (Schulz *et al*. 1994) and adapted for zebrafish testis explants. For these experiments, the two testes were immersed in Dulbecco PBS containing 1 mM $CaCl_2$ and $MgCl_2$ (D-PBS(+); Invitrogen, Carlsbad, CA, USA). One testis was incubated in basal medium (Leal *et al*. 2009b), serving as control for the contra-lateral testis, which was challenged with Igf1. Testis tissue was immersed in 200 µl medium in 96-well flat-bottom plates (Corning, New York, NY, USA) in a humidified air atmosphere at 25ºC for 16 h. After incubation, testis explants were weighed. The incubation medium was heated at 80°C for 1 h, centrifuged (16,000 g; 4°C) for 30 min and the supernatant was stored at -25°C for direct quantification of 11-KT levels by radioimmunoassay as reported (Schulz *et al*. 1994). The results were expressed as pg 11-KT released per mg of testis tissue. Long term effects on steroid release were evaluated on 6 zebrafish using the *ex vivo* organ culture technique, as described above. Incubation media and agarose cylinders were collected and stored at -25 ºC until extraction with 2x10 ml diethyl ether, in order to quantify 11-KT (Schulz *et al*. 1994). The results are expressed as pg 11-KT released per mg of testis tissue.

To examine if Igf1 and 11-KT have additive stimulatory effects to 11-KT on spermatogenesis, Tübingen AB strain zebrafish were used. One testis was incubated with 100 nM 11-KT (Leal *et al*. 2009b), while the contra-lateral was incubated with 100 nM 11-KT and 50 ng/ml recombinant gilthead seabream Igf1. Again, incubation took place in a humidified air atmosphere at 25ºC for 7 days, and medium was refreshed once after four days. Testis tissue samples were collected for histological and morphometrical analysis (n=8 explants per group).

Histological and morphometric analysis

Sample preparation and procedures for histology and morphometric analysis were conducted as described previously (Feitsma *et al*. 2007). Results were expressed as volume fractions (expressed as % of total tissue) and were determined for each of

the following testicular components: type A spermatogonia, type B spermatogonia, primary spermatocytes, secondary spermatocytes, spermatids, apoptotic germ cells, and others (including spermatozoa, somatic cells, blood and lymphatic vessels, connective tissue, and empty spaces). The morphological characteristics of the different zebrafish testicular cell types have been described in detail recently (Leal *et al*. 2009a).

BrdU-immunodetection and quantification of BrdU index

Zebrafish testis tissue explants were fixed for 5 h at room temperature in freshly prepared methacarn (60% [v/v] ethanol, 30% [v/v] chloroform, and 10% [v/v] acetic acid) and embedded in Technovit 7100 (Heraeus Kulzer). Three 5 µm thick sections per explant, collected at least 20 µm apart from each other, were subjected to BrdU immunodetection, as described previously (Leal *et al*. 2009b).

Visual inspection of the immunohistochemically processed sections revealed clearly elevated numbers of BrdU positive nuclei of type B spermatogonia and of spermatocytes in hormone-treated testis explants requiring no further quantification. However, the results were less clear as regards type A spermatogonia, so that we determined the BrdU labelling index of type A spermatogonia (*i.e.* BrdU positive percentage [%] of total number of type A spermatogonia). To this end, we quantified 4 microscopic fields (at 400X magnification) per section on 3 sections amounting to 12 observations per explant, which were averaged; seven explants were analysed per treatment.

Gene expression analysis

Testes collected for gene expression analysis were snap frozen in liquid nitrogen and stored at -80 ^{o}C until RNA extraction. Total RNA was extracted from testis samples using the RNAqueous-Micro Kit (Ambion, Austin, TX, USA), following the manufacturers' instructions. Further processing to estimate the relative mRNA expression levels of a number of selected genes (see below) by real-time, quantitative PCR was performed as described previously (De Waal *et al*. 2008). A commercially available TaqMan gene expression assay was acquired to detect the endogenous control RNA, eukaryotic 18S ribosomal RNA (18S rRNA; TaqMan gene expression assays; Applied Biosystems; Foster City, CA, USA). Primers (see Table 1) were designed for *igf1*, *igf2a*, *igf2b*, *igf3*, *igf1ra*, *igf1rb* and *piwil2* (a.k.a. *zili*; a germ cell marker; (Houwing *et al*. 2008), and were tested before use for specificity and amplification efficiency on serial dilutions of testis cDNA (results not shown), as described elsewhere (Bogerd *et al*. 2001). All qPCRs 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 (De Waal *et al*. 2008). Relative mRNA levels were calculated as reported (Bogerd *et al*. 2001).

Statistical analysis

For the *in vivo* E_2 exposure, differences in relative mRNA levels between treatments were tested using the Student unpaired t-test. For the *ex vivo* experiments, differences between treatments for the measured parameters (*i.e.* amount of androgens released, relative mRNA levels, volume densities, BrdU index of type A spermatogonia) were tested for statistical significance using the Student paired t-test for paired observations (*e.g.* basal versus gonadotropin, or trilostane versus trilostane + gonadotropin). Thereafter, values were expressed as fold of basal or control levels and significant differences between unpaired treatments (*e.g.* gonadotropin versus trilostane + gonadotropin) were identified using the Student unpaired t-test; comparison of more than two groups for *in vivo* and *ex vivo* experiments was performed with one-way ANOVA with Newman-Keuls testing. To allow the comparison of relative mRNA levels (fold of basal) of the different 11-KT concentrations, relative mRNA levels of the corresponding basal control groups were tested for significant differences as well. In some cases, data were log transformed to achieve an equal variance. Using GRUBBS software (GraphPad, San Diego, CA) possible outliers were identified. A significance level (P) of 0.05 was applied in all the statistical analyses, which were performed using the Prism4 software package (GraphPad).

Results

Expression of IGF peptide and IGF receptor mRNAs in zebrafish testis models

We compared the relative mRNA levels coding for the individual IGF peptides (*i.e. igf1*, *igf2a*, *igf2b*, *igf3*) and type 1 IGF receptors (*i.e. igf1ra* and *igf1rb*) between testes cultured *ex vivo* under basal conditions for 7 days and testes extracted directly from zebrafish (Fig. 1A). Among the ligands, only the *igf3* transcript levels decreased significantly in testes cultured *ex vivo* compared with the *in vivo* situation (Fig. 1A). The mRNA levels of both *igf1ra* and *igf1rb* decreased substantially (by ~2 and ~4 fold, respectively) under basal culture conditions compared with *in vivo* levels (Fig. 1A).

To study if the mRNAs for the IGF peptides and their type 1 receptors were expressed in somatic or germ cells in zebrafish testis, we examined *piwil1*$^{(-/-)}$ zebrafish, which present germ cell-depleted testes adults (Houwing *et al*. 2007).

This was confirmed by the very low mRNA levels of the germ cell marker *piwil2* (Houwing *et al*. 2008). In contrast, the *igf2b* and *igf1ra* transcript levels although being significantly lower than in wild type testis(Fig. 1B) were still clearly detectable in *piwil1*$^{(-/-)}$ testis. Moreover, the mRNA levels of the other IGF peptides and *igf1rb* were similar in mutant and wild type testis (Fig. 1B) suggesting that the bulk of IGF peptide and IGF receptor transcripts are expressed in the somatic cells of the testis.

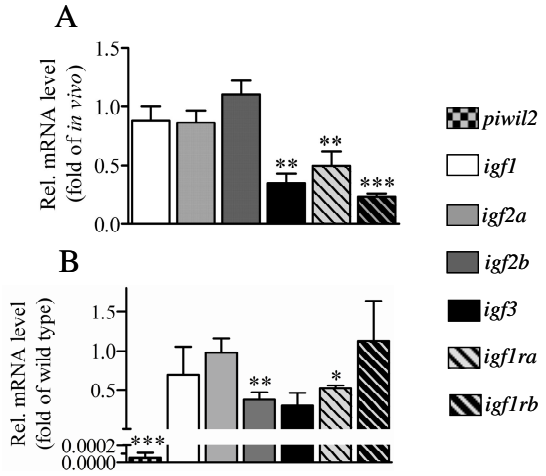

Figure 1. Messenger RNA expression of IGFs and type 1 IGF receptors in experimental zebrafish testis models. Relative mRNA levels of IGF ligands and IGF receptors in testicular explants cultured *ex vivo* for 7 days (n=38 explants) and in *piwil1*$^{(-/-)}$ testis (n=3 fish). Bars represent the mean (± SE) relative mRNA levels, normalised to 18S rRNA, and were expressed as fold of relative *in vivo* (n=28-29 fish; A) or wild type (n=3 fish; B) mRNA levels. Bars marked with ∗ (P<0.05), ∗∗ (P<0.01), or ∗∗∗ (P<0.0001) are significantly different (unpaired t-test) from *in vivo* (A) or wild type (B) levels.

Expression of igf3 mRNA in zebrafish Sertoli cells

In situ hybridisation localisation of the gonad-specific *igf3* mRNA revealed a positive signal in discrete intratubular cells scattered throughout the testicular parenchyma (Fig. 2A), while sections incubated with the sense *igf3* probe showed no specific signal (Fig. 2B). Based on the cell shape, size and localisation close to the basement membrane, the cells with a positive *in situ* hybridization signal were identified as Sertoli cells (Figs. 2C, 2D). Sertoli cells show kidney- or triangular-shaped nuclei, while germ cells have round/oval-shaped nuclei. However, more Sertoli cells are present in the tubules than the number of Sertoli cells showing a clear *in situ* hybridisation signal. (see for comparison Fig. 2E). The nuclear size, shape, diffuse distribution of the Nuclear Fast Red staining and number of germ

cell nuclei associated with those Sertoli cells showing a clear *in situ* hybridisation signal, suggest that these germ cells are type A spermatogonia.

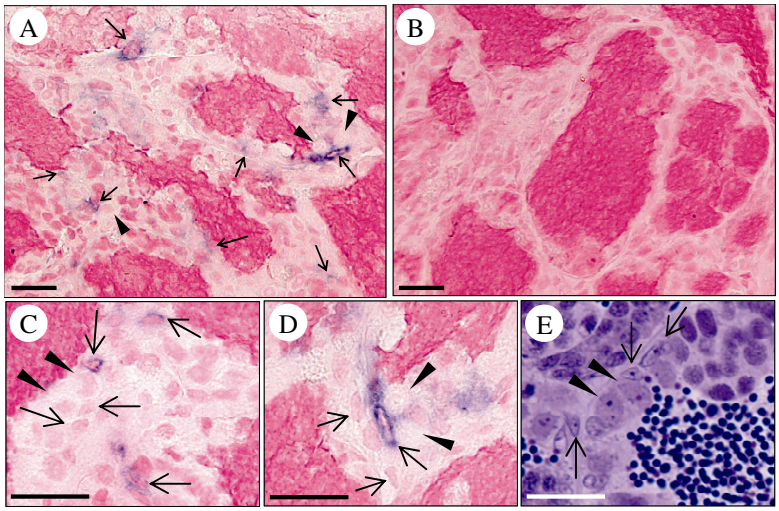

Figure 2. Localization of *igf3* mRNA in zebrafish Sertoli cells. Lower magnification (40x) of glycolmethacrylate embedded 7µm thick section of testis, hybridized with *igf3* (A) antisense or (B) sense probe. (C and D) Higher magnification (100x) of glycolmethacrylate embedded section of testis hybridized with *igf3* antisense probe, revealed cytoplasmic staining in subpopulation of Sertoli cells. Sections were counterstained with Nuclear Fast Red. (E) Glycolmethacrylate embedded histological (4µm thick) section stained with toluidine blue shows similar nucleus of Sertoli cell contacting A type spermatogonia. Indicated are the nuclei of Sertoli cells (arrows) and A type spermatogonia (arrowheads), while spermatozoa are indicated with Sz (in E, F and G). Scale bars = 20µm.

Sex steroid modulation of IGF mRNA levels in zebrafish testis

IGF signalling has been shown to either stimulate fish spermatogonial proliferation directly (Loir 1994) or to permissively enable androgen-stimulated spermatogonial proliferation (Nader *et al*. 1999). Here, we studied aspects of the endocrine regulation of IGF mRNA levels.

Testis explants incubated with 11-KT displayed a dramatically increased number of differentiating germ cells (*i.e.* type B spermatogonia, spermatocytes and spermatids) compared with explants cultured under basal conditions where type A spermatogonia and spermatozoa predominated (Figs. 3A, 3B). Detection of the proliferation marker BrdU, added during the last 24 h of culture, demonstrated a stimulatory effect of 11-KT on the proliferation of type A spermatogonia (inset Fig. 3A), next to a very clear stimulation of DNA synthesis in type B spermatogonia and spermatocytes (Fig 3B). In testis explants, incubated with increasing concentrations of 11-KT, no systematic changes were observed as regards *igf1, igf2a* and *igf2b* mRNA levels. In contrast, the *igf3* mRNA levels were elevated to a

maximum of 5-fold compared with basal levels in response to 11-KT (Fig. 3C). Elevations in *igf3* expression did not differ significantly between concentrations (ANOVA, Student Newman-Keuls test). Recently, we have shown that oestrogen exposure can cause a state of androgen insufficiency in zebrafish testis *in vivo* associated with an inhibition of spermatogonial proliferation and differentiation (De Waal *et al*. 2009). Exposure to E_2 *in vivo* for 6 days reduced testicular *igf3* mRNA levels down to ~18% of control levels, whereas *igf1* mRNA levels again showed no change. In contrast, the *igf2a* and *igf2b* mRNA levels rose after E_2 exposure *in vivo* up to 2.8 fold of control levels (Fig. 3D).

Direct or sex-steroid-mediated gonadotropin modulation of IGF mRNA levels in zebrafish testis

To investigate possible gonadotropic regulation of testicular IGF mRNA expression *in vivo*, zebrafish received an intraperitoneal gonadotropin injection. Two hours after injection, both Fsh and Lh induced significant rises in testicular *igf1* mRNA levels, Fsh being more potent than Lh (Fig. 4). Where *igf2a, igf2b* and *igf3* transcript levels did not change significantly after Lh injection, *igf2b* expression decreased significantly following Fsh injection. Another difference between the two gonadotropins was that Fsh, but not Lh, induced a strong (~20-fold) up-regulation of *igf3* mRNA levels *in vivo* (Fig. 4).

To dissect the mechanisms of gonadotropin regulation on IGF mRNAs, testis explants were incubated with gonadotropin in the absence or presence of the 3β-HSD inhibitor, trilostane. Neither Lh nor Fsh treatment caused an alteration in the *igf1* and *igf2b* mRNA levels, while the *igf2a* mRNA levels declined significantly after Fsh treatment. However, the *igf3* mRNA levels increased after Fsh (~7 fold) and Lh (~2 fold) treatment above basal levels (Figs. 5A, 5B). Whereas Lh-mediated induction of *igf3* expression was reduced even below basal levels, the Fsh-mediated up-regulation of *igf3* mRNA levels was not prevented by co-incubation with trilostane (Figs. 5A, 5B).

Recombinant fish Igf1 supports zebrafish spermatogenesis without modulating steroidogenesis

To investigate the effect of Igf1 signalling on zebrafish spermatogenesis, testis explants were incubated in the presence of increasing concentrations of recombinant gilthead seabream Igf1. Histological evaluation showed an increasing abundance of more differentiated germ cell stages, such as type B spermatogonia, spermatocytes and spermatids, with increasing Igf1 concentrations (Fig. 6A-H).

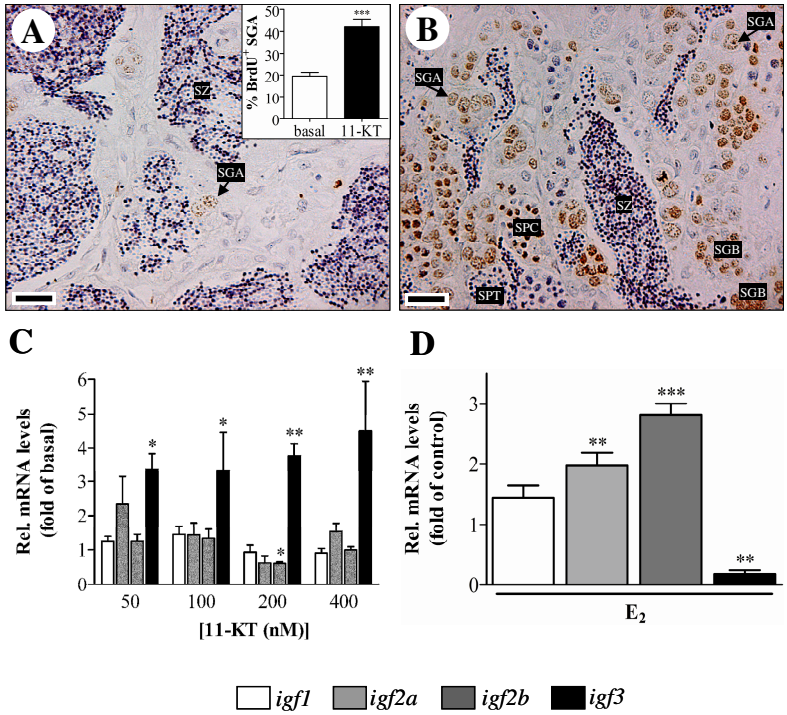

Figure 3. Sex steroid regulation of zebrafish spermatogenesis and IGF mRNAs. BrdU labelling in testis explant cultured *ex vivo* under basal conditions (A) or in the presence of 400 nM 11-KT (B). (Inset A) Quantitative analysis of the percentage (%) of BrdU-positive nuclei of type A spermatogonia (n=6 explants per treatment). BrdU was present during the last 24 hours of culture. Note the BrdU-labelled type A spermatogonia (SGA), type B spermatogonia (SGB) and spermatocytes (SPC) in (B), whereas spermatids (ST) and spermatozoa (SZ) remained unlabelled. Scale bars = 20 µm. Relative mRNA levels of zebrafish IGFs in (C) testicular explants treated with increasing concentrations of 11-KT *ex vivo* (n=4-7 explants per dose) for 7 days, and in (D) testis of zebrafish exposed to 10 nM E_2 *in vivo* (n=12 fish per treatment) for 6 days. Bars represent the mean (± SE) relative mRNA levels, normalised to 18S rRNA, and were expressed as fold of basal (*ex vivo*; C) or control (*in vivo*, D) relative mRNA levels. Bars marked with ∗ (P<0.05), ∗∗ (P<0.01), or ∗∗∗ (P<0.0001) are significantly different (*ex vivo*, C, paired t-test; *in vivo*, D, unpaired t-test) from control or basal. No significant differences were found in relative IGF mRNA levels of the corresponding basal control groups and their responses to 11-KT between the different concentrations (ANOVA, Student Newman-Keuls test; P<0.05).

Since the otherwise favoured pair-wise correlation of the two testes of one animal divided over treatments was not possible because of technical problems, we compared the different treatment groups with an unpaired t-test. Morphometrical analysis showed that the volume fractions of type B spermatogonia, primary spermatocytes and spermatids increased significantly at 25 ng/ml Igf1 (Fig. 6I). At a concentration of 50 ng/ml Igf1, primary spermatocytes and spermatids were still elevated but now also secondary spermatocytes were found in increased volumes, while type B spermatogonia fell back to basal levels (Fig. 6I). Apoptotic germ cells and other testicular cell types showed no change in all treatments and explants

exposed to 12.5 ng/ml Igf1 were similar to basal regarding cellular composition (Fig. 6I). To evaluate whether an exposure at 50 ng/ml Igf1 affected spermatogenesis directly or rather via increased synthesis of androgens, we determined the release of 11-KT overnight, after the first half, and after the second half of long term culture (*i.e.*, 16 h, 0-3.5 d, 3.5-7 d, respectively). At none of the timepoints, the 11-KT release was modulated by Igf1 (Fig. 7).

Since a permissive role of Igf1 to androgen-stimulated spermatogenesis was reported for eel testis explants (Nader *et al*. 1999), we tested the possibility that Igf1 and 11-KT interact. Although qualitative histological assessment did not suggest obvious interaction effects on spermatogenesis (Fig. 8A-D), morphometric quantification of germ cell type volume fractions revealed declines in the abundance of type A and B spermatogonia, and of primary spermatocytes, whereas other testicular cell types (for ~80% consisting of spermatozoa) increased significantly ($P<0.05$) (Fig. 8E). These results suggest that the combination of Igf1 and androgen stimuli resulted in more spermatogonia passing through meiosis and spermiogenesis.

Discussion

The role of IGF signalling in male reproduction has been studied extensively in mammals (*e.g.* Lin *et al*. 1986; Söder *et al*. 1992; Baker *et al*. 1996; Wang *et al*. 2003; Froment *et al*. 2004, 2007; Wang and Hardy 2004). However, many aspects of IGF functions in male reproduction of fish have not received much attention yet. In pubertal chinook salmon (*Oncorhynchus tshawytscha*), Igf1 plasma levels peaked during early spermatogonial proliferation and reached maximum levels in spermiating males (Campbell *et al*. 2003). Moreover, recombinant human IGF1 stimulated salmonid spermatogonial proliferation *in vitro* (Loir and Le Gac 1994; Loir 1999) and permissively enabled the progression of eel spermatogenesis in long-term testis tissue cultures incubated with 11-KT (Nader *et al*. 1999). However, in an attempt to elucidate aspects of paracrine IGF action on adult fish spermatogenesis, the present study is, to our knowledge, the first to combine localization of testicular expression, and gonadotropic and sex steroid regulation of zebrafish IGF ligands and type 1 IGF receptors with analysis of IGF bioactivity on adult spermatogenesis in fish. Analysis of germ cell-depleted zebrafish testis showed that all IGFs and IGF type 1 receptors were expressed predominantly in the somatic compartment. This is line with previous observations in different teleost species (Le Gac *et al*. 1996; Reinecke *et al*. 1997; Perrot *et al*. 2000; Berishvili *et*

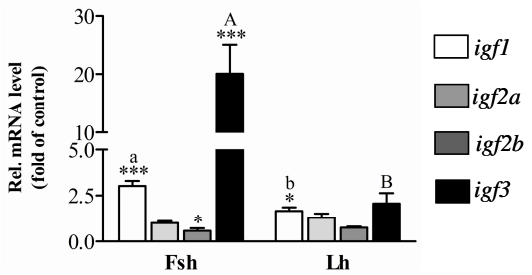

Figure 4. Gonadotropin regulation of IGF mRNAs in zebrafish testis *in vivo*. Testicular relative (Rel.) IGF mRNA levels 2 h after injection of 100 ng/g recombinant zebrafish Fsh or 100 ng/g recombinant zebrafish Lh (n=6-8 fish per treatment). Bars represent the mean (± SE) relative IGF mRNA levels, normalised to 18S rRNA, and data were expressed as fold of control (PBS-injected fish). Bars marked with ∗ (P<0.05), ∗∗ (P<0.01), or ∗∗∗ (P<0.001) are significantly different (ANOVA, Student Newman-Keuls test) from control condition, and bars marked with different letters denote statistical significant differences between the Fsh and Lh response (ANOVA, Student Newman-Keuls test; P<0.05).

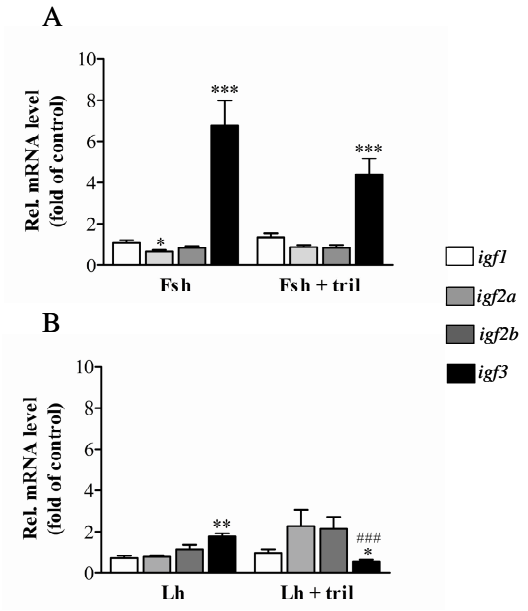

Figure 5. Gonadotropin regulation of IGF mRNAs in zebrafish testis *ex vivo*. Relative (Rel.) IGF mRNA levels after 2 days exposure to (A) 100 ng/ml recombinant zebrafish Fsh (n=6-8 explants per treatment) or (B) 500 ng/ml recombinant zebrafish Lh (n=7-8 explants per treatment) alone and in combination with 25 µg/ml of trilostane (tril). Bars represent the mean (± SE) relative mRNA levels, normalised to 18S rRNA. Data were expressed as fold of control (basal or tril) relative mRNA levels. Bars marked with ∗ (P<0.05), ∗∗ (P<0.01), or ∗∗∗ (P<0.0001) are significantly different (paired t-test) from their respective control condition, and bars marked with ### (P<0.0001) are significantly different (unpaired t-test) from the respective gonadotropin-induced relative mRNA level without trilostane co-incubation.

al. 2006; Wang *et al*. 2008). The significant decrease in *igf2a* and *igf1ra* mRNA level in *piwil1*$^{(-/-)}$ testis compared with wild type testis could indicate germ cell expression of these genes as well as observations of Igf1 and/or Igf1r immunoreactivity have been reported for Mozambique tilapia (*Oreochromis mossambicus*) spermatocytes and gilthead seabream spermatogonia and spermatids (Reinecke *et al*. 1997; Perrot *et al*. 2000). The gonad-restricted expression of the teleost-specific *igf3* mRNA (Wang *et al*. 2008) renders it an interesting candidate for studies on paracrine IGF action. There is limited information on the cellular localization of transcript or protein in testis, since *igf3* sequences have been found in only a few teleost species (zebrafish, medaka [*Oryzias latipes*] and Nile tilapia [*Oreochromis niloticus*]; Wang *et al*. 2008). These authors reported the presence of *igf3* mRNA in interstitial cells of adult Nile tilapia testis. Our *in situ* hybridisation analysis in zebrafish, however, suggests *igf3* mRNA expression by Sertoli cells (Fig. 2) while interstitial areas remained unlabeled. Detection of IGF mRNA or protein in Sertoli cells is consistent with previous observations in different vertebrate species (Tres *et al*. 1986; Vanelli *et al*. 1988; Hansson *et al*. 1989; Cailleau *et al*. 1990; Naville *et al*. 1990; Dombrowicz *et al*. 1992; Froment *et al*. 2007) including species with cystic spermatogenesis, such as rainbow trout (*Oncorhynchus mykiss*), Mozambique tilapia, gilthead seabream, newt (*Cynops pyrrhogaster*) and shark (*Squalus acanthias*) (Dubois and Callard 1993; Le Gac *et al*. 1996; Reinecke *et al*. 1997; Perrot *et al*. 2000; Yamamoto *et al*. 2001; Li *et al*. 2008). In the latter species, cultured spermatogenic cysts consisting of Sertoli cells surrounding spermatogonia secreted high levels of Igf1 (Dubois and Callard 1993). Taken together, Sertoli cells appear to be an important site of IGF release in the vertebrate testis and it is, therefore, conceivable to consider the zebrafish Sertoli cell as an important source of Igf3 secretion.

Gonadotropins modulate testicular IGF synthesis and secretion in mammals and amphibians (Cailleau *et al*. 1990; Naville *et al*. 1990; Yamamoto *et al*. 2001). For example, FSH increased *igf1* mRNA levels in newt testicular organ culture (Yamamoto *et al*. 2001), or stimulated IGF1 secretion from rodent and porcine Sertoli cells *in vitro* (Cailleau *et al*. 1990; Naville *et al*. 1990). *In vivo*, combined androgen/FSH or FSH treatment alone increased intra-testicular IGF1 protein levels in hypophysectomised and GnRH-antagonist-treated rats (Spiteri-Grech, *et al*. 1993; Itoh *et al*. 1994). In fish, information on this subject is not available. In the present study, we have demonstrated differential effects of androgens and gonadotropins on testicular mRNA levels of IGF peptides. Gene expression of *igf3* and both receptor forms were downregulated in the absence of stimulating factors in tissue culture. While *igf1ra* and *igf1rb* mRNA levels were not restored by

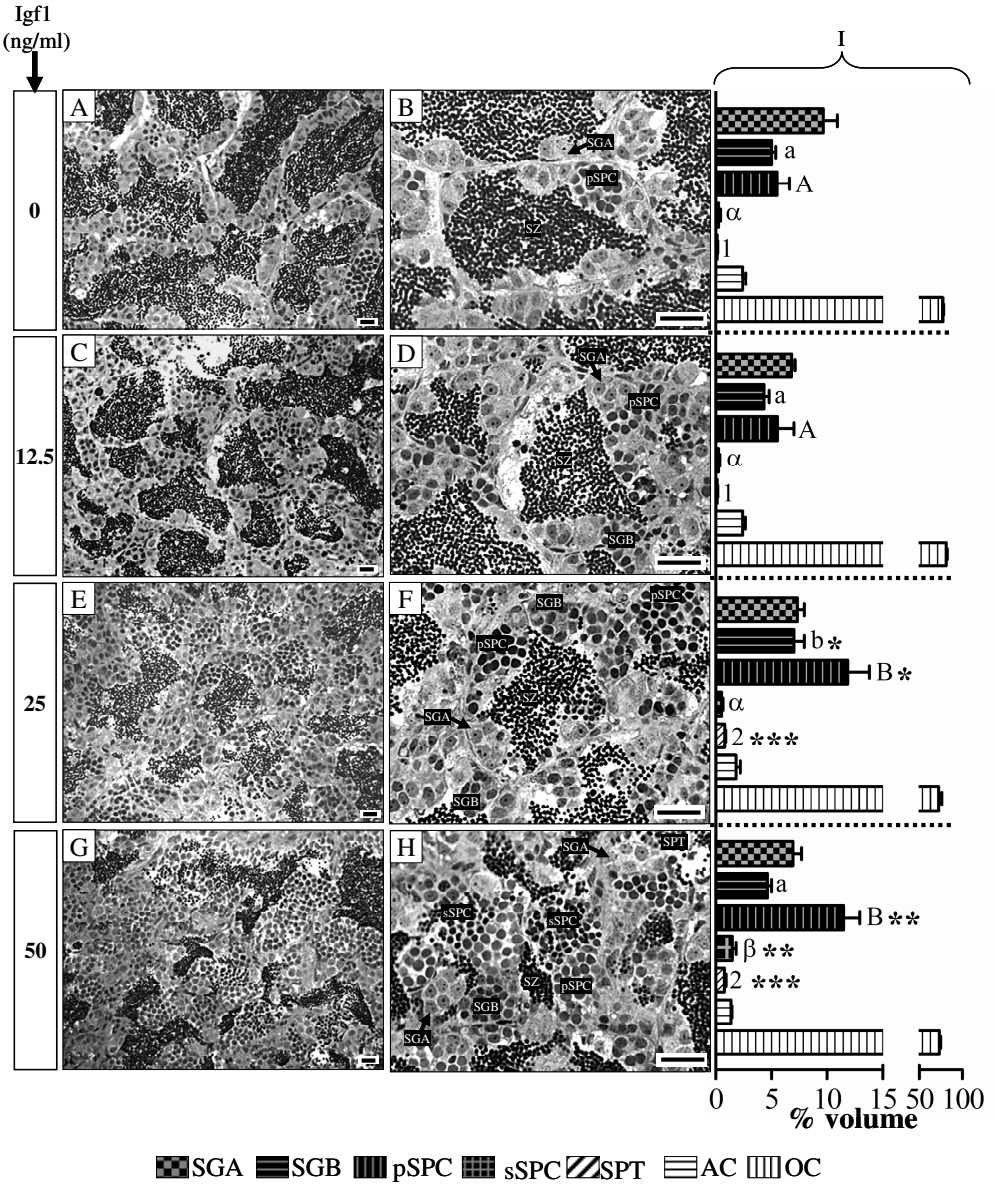

Figure 6: Effects of Igf1 on zebrafish spermatogenesis. Zebrafish testis sections from explants exposed to 0 (basal; A and B), 12.5 (C and D), 25 (E and F) and 50 (G and H) ng/ml recombinant gilthead seabream Igf1 *ex vivo*. Photographs were taken at low (A, C, E and G) and high (B, D, F and H) magnification. Scale bar = 20 µm. (I) Morphometric analysis (n=5-6 explants per dose) of zebrafish testis sections, presenting data as testis tissue volume percentage. Bars marked with ∗ (P<0.05), ∗∗ (P<0.01), or ∗∗∗ (P<0.0001) are significantly different from basal; each parameter in panel (I) indicated with different letters or numbers denote statistical differences (all one-way ANOVA; Newman-Keuls test; P<0.05). Abbrevations: type A spermatogonia, SGA; type B spermatogonia, SGB; primary spermatocytes, pSPC; secondary spermatocytes, sSPC; spermatids, SPT; spermatozoa, SZ; apoptotic germ cells, AC; other testicular cell types, OC.

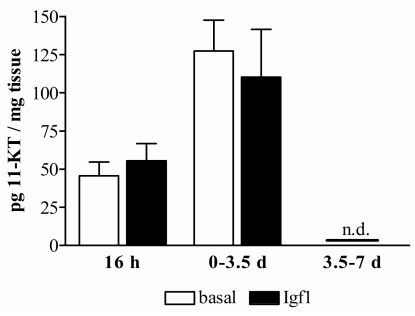

Figure 7: Effect of Igf1 on zebrafish testicular steroidogenesis. Measurement of testicular 11-KT release after acute (16 h; n=11 explants per treatment), medium term (0-3.5 d; n=6 explants per treatment) or long term (3.5-7 d; n=6 explants per treatment) exposure to 50 ng/ml recombinant gilthead seabream Igf1. Bars represent the mean (± SE) androgen release, expressed as pg 11-KT released per mg testis tissue. No statistical significant differences between treatments were found using Student Paired t-testing (P<0.05).

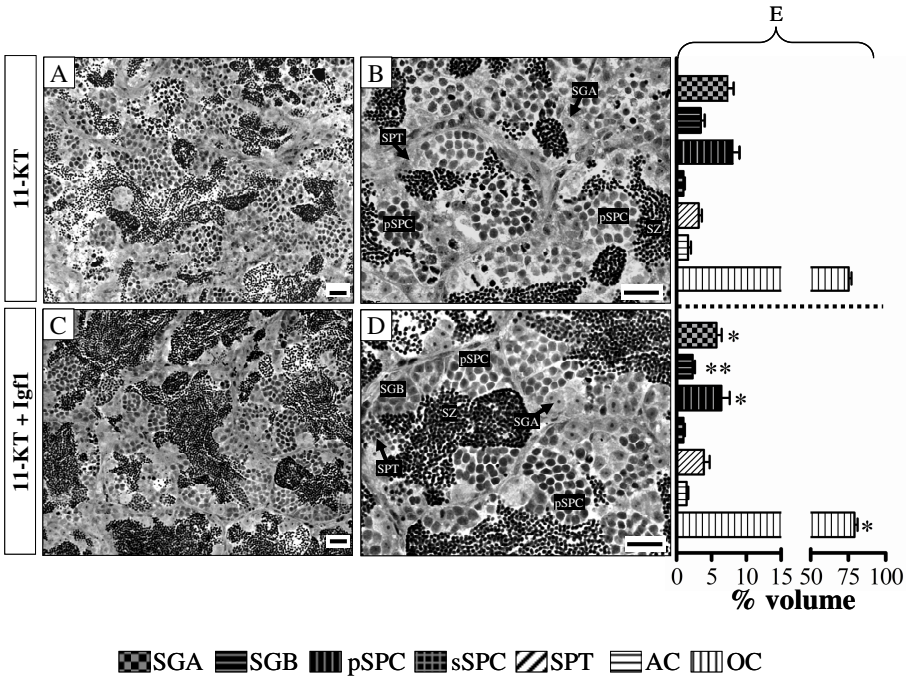

Figure 8: Additive Igf1 effect on androgen-stimulated spermatogenesis. Zebrafish testis sections from explants exposed to 11-KT alone (A and B) and 11-KT + 50 ng/ml recombinant gilthead seabream Igf1 (C and D). Photographs were taken at low (A and C) and high (B and F) magnification. Scale bar = 20 µm. (E) Morphometric analysis (n=8 explants per treatment) of zebrafish testis sections, presenting data as testis tissue volume percentage. Bars represent the mean (± SE) testis tissue volume density (%). Bars marked with ∗ (P<0.05), ∗∗ (P<0.01) are significantly different from 11-KT alone (Student Paired t-test). Abbrevations: type A spermatogonia, SGA; type B spermatogonia, SGB; primary spermatocytes, pSPC; secondary spermatocytes, sSPC; spermatids, SPT; spermatozoa, SZ; apoptotic germ cells, AC; other testicular cell types, OC.

androgen treatment in the same organ culture system (data not shown), *igf3* mRNA levels were restored to *in vivo* levels either by direct androgen exposure (Fig. 3C) or by gonadotropin-induced androgen release. In this regard, Fsh was much more potent than Lh (see below). Moreover, changing androgen plasma levels *in vivo*, either lowered levels in oestrogen-induced androgen insufficiency model, or increased following Fsh or Lh injections, modulated *igf3* mRNA levels accordingly. However, also in the presence of trilostane and the subsequent block of production of biologically active androgen, Fsh, but not Lh, still clearly up-regulated *igf3* expression. The strong up-regulation of *igf3* expression after Fsh treatment *in vivo* could therefore be a result of contributions of both androgen-independent and -dependent Fsh action (Fig. 4). In this regard, suppression of Fsh synthesis and/or release caused by negative oestrogenic feedback has been reported in other species (Dickey and Swanson 1998; Kobayashi *et al*. 2001; Huggard-Nelson *et al*. 2002) and could also have contributed to the down-regulation of *igf3* mRNA levels in androgen-insufficient zebrafish. The differential gonadotropin-mediated modulation of *igf1* mRNA levels *ex vivo* (*i.e.* no change) compared with *in vivo* (*i.e.* increased after Fsh and Lh injection) could be due to short-term changes in *igf1* expression, since the *in vivo* effect was evident after only 2 hours, whereas *ex vivo* gonadotropin exposure lasted 2 days. There was no consistent pattern as regards *igf2a* or *igf2b*, and changes were of comparatively low amplitude. Moreover, *igf2a* and *igf2b* mRNA levels in testis tissue remained stable during *ex vivo* culture (*i.e.* isolated from gonadotropic input and with quickly decreasing activity of the steroidogenic system) suggesting that expression of these peptides is regulated by other mechanisms.

In zebrafish, oestrogen-induced androgen insufficiency resulted in reduced proliferation and differentiation of type A spermatogonia *in vivo* (De Waal *et al*. 2009). The present study showed that 11-KT increased the mitotic index of type A and B spermatogonia *ex vivo* (Fig. 3A, 3B), previous work showed that 11-KT increased the incidence of BrdU-labelled spermatids and spermatozoa in zebrafish testis tissue culture *ex vivo* (Leal *et al*. 2009b). Taken together, these data suggest that in zebrafish androgens support all three main phases of adult spermatogenesis: the mitotic, meiotic, and the spermiogenic phase. This is in accordance with previously published data in juvenile Japanese eel, where 11-KT induced full spermatogenesis associated with down-regulation of the expression of an inhibitory (Miura *et al*. 2002) and up-regulation of a differentiation-promoting (Miura *et al*. 1995) TGFβ-family member. In mammals, on the other hand, the stimulatory effects of androgens on the spermatogonial phase is much less prominent, but increased with advancing differentiation of the germ cells (*e.g.* El

Shennawy *et al*. 1998; França *et al*. 1998). The latter studies addressed the point that in addition to androgens, also FSH regulates spermatogenesis. In fish, however, Fsh is a strongly steroidogenic hormone (*e.g.* (Planas and Swanson 1995; Zmora *et al*. 2007; Kazeto *et al*. 2008; García-López *et al*. 2009, in preparation), Fshr expression has been localised in Leydig cells (Ohta *et al*. 2007; García-López *et al*. 2009, in preparation;), and Fsh-stimulated androgen production is sufficient to stimulate complete spermatogenesis in juvenile eel testis *ex vivo* (Ohta *et al*. 2007). Hence, steroid-independent effects of Fsh on testis physiology have not been characterised yet in fish. Interestingly, Fsh stimulated proliferation of spermatogonia in rainbow trout primary testicular cell cultures consisting mainly of Sertoli and germ cells (Loir 1999). Considering the mitogenic action of IGF peptides on germ cells in other anamniote species (Dubois and Callard 1993; Loir 1994, 1999; Loir and Le Gac 1994; Nakayama *et al*. 1999; Li *et al*. 2008), and considering the high *igf3* mRNA expression in Sertoli cells and its regulation by androgens and Fsh, we hypothesise that *igf3* plays a role in the proliferation and/or differentiation of spermatogonia.

Although in mammalian vertebrates a cation-independent mannose 6-phosphate receptor (MPR) shows high affinity to IGF2, in non-mammalian vertebrates this seems not to be the case (Canfield and Kornfeld 1989; Clairmont and Czech 1989; Yang *et al*. 1991). This suggests that the type 1 IGF receptors are the main signalling partners for all IGF peptides in the zebrafish testis. Diversity exists between amino acid sequences of Igf3 and other zebrafish IGFs in their C domain, which is involved in high affinity binding of IGFs to the type 1 IGF receptors (Bayne *et al*. 1989), so that a presently unknown, novel Igf3 receptor might exist. Alternatively, the conservation of residues in the A and B domain, which are essential to receptor binding and activation among all IGF peptides, could allow Igf3 interaction with the type 1 IGF receptors (Wang *et al*. 2008), and akin biological functions among IGF ligands were suggested based on proposed similar tertiary structures (Wang *et al*. 2008). Efforts are being made at present to produce recombinant zebrafish Igf3.

To elucidate aspects of IGF bioactivity on zebrafish spermatogenesis, we have exposed testis explants to increasing concentrations of recombinant gilthead seabream Igf1. Concentrations of 25 ng/ml Igf1 were able to support spermatogenesis, which was reflected in a higher amount of differentiating germ cells (*i.e.* type B spermatogonia, spermatocytes and spermatids) (Fig. 6). These stimulatory concentrations of Igf1 are likely to be of physiological relevance, since Igf1 plasma levels measured in male salmonids ranged from 14 ng/ml at the start of gonadal maturation to 50 ng/ml during spermiation (Campbell *et al*. 2003; Onuma *et al*. 2009). The morphometric evaluation indicates that Igf1 stimulates

both spermatogonial proliferation and the entry of spermatogonia into meiosis and spermiogenesis. This is consistent with observations in cultures of isolated testicular cells, spermatocysts, seminiferous tubules and testis tissue in other species, where Igf1 stimulated DNA synthesis in spermatogonia *in vitro* (Söder *et al*. 1992; Dubois and Callard 1993; Loir 1994, 1999; Loir and Le Gac 1994; Li *et al*. 2008), or entry into meiosis in amphibian primary cell and organ culture (Nakayama *et al*. 1999; Li *et al*. 2008). Igf1 action on spermatogonia was mediated by Sertoli cells (Li *et al*. 2008), and IGF1R/*igf1r* protein or mRNA expression was detected in Sertoli-cell-enriched cell fractions (Le Gac *et al*. 1996; Yamamoto *et al*. 2001; Froment *et al*. 2007). Moreover, auto-upregulation of *igf1* was observed in newt testis (Yamamoto *et al*. 2001). Taken together, this suggests an autocrine mechanism of IGF action on zebrafish Sertoli cells as has been proposed previously for rodent Sertoli cells (Froment *et al*. 2007).

Although Igf1 was reported to stimulate steroidogenesis and may therefore stimulate spermatogenesis via androgens (Gelber *et al*. 1992; Chuzel *et al*. 1996; Lin *et al*. 1998), the present study did not provide evidence for the relevance of this pathway in zebrafish.

In pre-pubertal eel testis explants, recombinant human IGF1 alone could not induce spermatogenesis, while the growth factor was required for a permissive effect on androgen-stimulated spermatogenesis (Nader *et al*. 1999). Since zebrafish spermatogenesis can be supported *ex vivo* solely by androgens (Leal *et al*. 2009b), or by solely Igf1 (this study), it was interesting to investigate the combined effects of these compounds. The observed decreases in spermatogonial and spermatocytic volume fractions coincided with increased volume fraction of post-meiotic cell types. Therefore the combined presence of Igf1 and androgens might be strong differentiation signals leading to an accumulation of haploid cells. A possible mechanism how Igf1 could facilitate the additional differentiation effect of androgens on zebrafish spermatogenesis (or the permissive effect on androgen-stimulated spermatogenesis in pre-pubertal eel testis (Nader *et al*. 1999) is that IGF1 signalling inactivated FOXO1, a co-repressor of AR (Fan *et al*. 2007).

In conclusion, we have shown an important function of IGF signalling in zebrafish testis. Gonadotropins and sex steroids control mRNA expression of IGF peptides differentially. The recently detected, novel family member, *igf3*, was most consistently stimulated Fsh and/or androgens. Studies investigating recombinant zebrafish Igf3 pharmacology on the IGF receptors and its bioactivity on fish spermatogenesis are highly warranted. Using (for the time being) recombinant teleost Igf1, we could show for the first time that IGF supports the complete

process of adult fish spermatogenesis *ex vivo*, which was not mediated by androgens (*i.e.* Igf1 stimulation of steroidogenesis).

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6

Summarizing Discussion

Societal and translational significance of studies into regulation of adult fish spermatogenesis

Spermatogenesis is a tightly controlled process characterised by a close association of germ cells and Sertoli cells. These Sertoli cells provide an appropriate microenvironment for germ cells either to remain stem cells, or to execute a differentiation pathway, known as spermatogenesis. Sertoli cell activities are regulated by hormones, such as FSH and androgens. However, the mechanisms of regulation by FSH and androgens on adult spermatogenesis are still poorly understood.

There are different aspects of societal relevance to this subject. For example, the development of new male hormonal contraception strategies could benefit when new aspects of the regulation of adult spermatogenesis were unravelled. Similarly, new therapeutic avenues could be taken for infertility patients. With regard to fish spermatogenesis, knowledge on basic endocrine regulation of spermatogenesis could help developing measures against precocious puberty typically observed in fish kept in aquaculture, since sexual maturation reduces food conversion into body growth and increases susceptibility to disease.

In the studies presented in this thesis, zebrafish served as experimental model. Although differences exist in the Sertoli cell-germ cell associations between fish and mammalian spermatogenesis, the majority of the hormones, the hormone receptors and their activities are well-conserved (McGonnell and Fowkes 2006). Nevertheless, genome duplication events early in piscine evolution (Laudet 1997; Douard *et al*. 2008) and teleost-specific steroid hormones (*e.g*. 11-KT) (Schmidt and Idler 1962; Borg 1994) are indications for the complexity and specificity of the teleost reproductive system.

The present thesis aimed to obtain more knowledge on hormone-dependent phases and the mechanisms involved in the endocrine regulation of adult spermatogenesis. Publications describing the steroidogenic potency of several piscine Fsh proteins (Planas and Swanson 1995; Kazeto *et al*. 2008; García-López *et al*. 2009), including zebrafish Fsh (García-López *et al*. in preparation) and the expression of Fshr/*fshr* mRNA and protein in (zebrafish) Leydig cells (Ohta *et al*. 2007; García-López *et al*. 2009, in preparation) suggest that a significant part of Fsh action is mediated by androgens.

Androgen receptor signalling in zebrafish testis

Information on the protein mediating androgen effect, the zebrafish Ar, was missing. Therefore, the studies described in **Chapter 2** set out to characterise the pharmacology and expression patterns of the zebrafish Ar in testis, and to identify their main physiological ligands.

The cloning of the zebrafish *ar* has been described by others a little earlier (Jørgensen *et al.* 2007; Hossain *et al*. 2008). Although two distinct *ar* genes have been cloned from several fish species (Ikeuchi *et al*. 1999, 2001; Takeo and Yamashita 1999, 2000; Katsu *et al*. 2007), Southern blot analysis by Hossain and colleagues (2008) indicated the presence of only a single *ar* gene in the zebrafish genome. This was consistent with a recent study by Douard and colleagues (2008) on the evolution of piscine *ar* genes, which described the loss of the duplicated zebrafish *ar* gene (*arb*).

The typical fish androgen, 11-ketotestosterone (11-KT), is proposed to be the main physiologically relevant ligand for the zebrafish Ar. Our androstenedione (A_2) metabolism experiments indicated that 11-KT was the major end product in zebrafish testis and 11-KT levels in serum are also high in closely related species, such as carp and goldfish; T was produced in low amounts in the zebrafish testis. However, high T serum levels were found in carp and goldfish (Rosenblum *et al*. 1985; Koldras *et al*. 1990). Furthermore, high affinity of T, but not of 11-KT, to serum hormone binding globulin (Shbg) in sea bass indicate a longer half-life of T in circulation (Miguel-Queralt *et al*. 2004). The prolonged presence in plasma suggests T may play specific roles in zebrafish as well. The zebrafish Ar did not distinguish between 11-KT and T in terms of binding and activation, which was consistent with other studies (Jørgensen *et al*. 2007; Hossain *et al*. 2008). However, these studies did not combine a detailed comparison of the ligand binding characteristics and the transactivation potentials of the zebrafish Ar as shown in **Chapter 2**. Both androgens had high binding affinities to Ar and could activate Ar-mediated transcription of an androgen-responsive promoter fused to a reporter gene at similarly low concentrations and with high potencies.

This raises an interesting question on how T and 11-KT may have distinct actions in adult fish testis. For example, in adult African catfish (*Clarias gariepinus*), T exerts negative feedback on testicular androgen production *in vitro*, different from 11-KT, of which transient negative feedback effects disappear after puberty (Schulz *et al*. 2008). In this way, T prevented 11-KT stimulation of spermatogenesis by 11-KT in African catfish (Cavaco *et al*. 2001) supposedly by impairment of Leydig cell function (Cavaco *et al*. 1999). Furthermore, 11-KT, but not T, induces

spermatogenesis in Japanese eel (*Anguilla japonica*) testis culture and in African catfish *in vivo* (Cavaco *et al*. 1998; Miura *et al*. 1991). However, only a single *ar* gene exists in zebrafish. The existence of a single *ar* gene was also suggested for the Siluriform order, to which African catfish belongs (Douard *et al*. 2008). So how can T and 11-KT exert differential biological activities in conjunction with the presence of only one non-discriminating Ar? A hypothesis is that conformational changes in the Ar protein upon binding by T or 11-KT are distinct, as has been suggested recently for T and 5α-dihydrotestosterone, a mammalian androgen binding to the mammalian AR (Askew *et al*. 2007). This could result possibly in interactions to differential coregulator proteins, and could, therefore, lead to alternate biological responses between ligands. In addition, differential spatio-temporal expression patterns of *ar* and its vast network of coregulators (Bebermeier *et al*. 2006), even within the testis (Domanskyi *et al*. 2007; Shan *et al*. 1995), could potentially modify the cell type- and tissue-specific biological responses to androgens.

Thus, our studies continued with the investigation of spatial *ar* expression within the adult zebrafish testis. By *in situ* hybridisation analysis on testis cryosections, no *ar* mRNA was detected in the interstitial compartment, whereas high *ar* mRNA levels were detected in Sertoli cells. The prevalence of *ar* mRNA to Sertoli cells is consistent with ~11-fold higher *ar* mRNA levels in laser-dissected intratubular areas (IT) as compared with interstitial areas (IS) isolated from zebrafish cryosections (Fig. 1). To identify interstitial areas, 3β-HSD staining was performed on zebrafish testis cryosections of 5 µm thickness according to previously described methods (García-López *et al*. 2009). The evaluation of the purity of the fractions revealed ~11-fold higher *gsdf* (*i.e.* a Sertoli cell-specific gene (Sawatari *et al*. 2007)) mRNA levels in IT, whereas ~300 fold lower *rlx3c* (*i.e.* Leydig cell-specific gene) mRNA levels were detected in IT. This finding is compatible with a small impurity of Sertoli cells in IS (García-López *et al*. in preparation), which may also contribute to the appararent *ar* mRNA expression in the IT fraction.

Not all zebrafish Sertoli cells express equal amounts of *ar* mRNA; rather a subpopulation of Sertoli cells contacting type A spermatogonia expresses the highest *ar* mRNA levels. In mature Atlantic cod (*Gadus morhua*) testis, *ar* was expressed by Sertoli cells as well; more specifically, *ar* mRNA is expressed at its highest level in Sertoli cells enveloping cysts of rapidly proliferating spermatogonia, and decreased when spermatogenesis progressed through meiosis, whereas no *ar* mRNA was detected surrounding spermatids (Almeida *et al*. 2009). Similarly, rodent Sertoli cells did not all express *Ar* mRNA at a similar level in

adult testis; rat Sertoli cells expressed *Ar* mRNA increasingly from stage IV to stage VII-VIII, after which expression decreased in stage IX-XI (Shan *et al*. 1995).

Observations that androgens stimulate spermatogenesis *in vivo* (Cavaco *et al*. 1998) and *in vitro* (Miura *et al*. 1991) in prepubertal fish, and the presence of *ar* mRNA in Sertoli cells enveloping spermatogonia, raised our interest into the development of experimental models to investigate the role of androgens in adult zebrafish spermatogenesis.

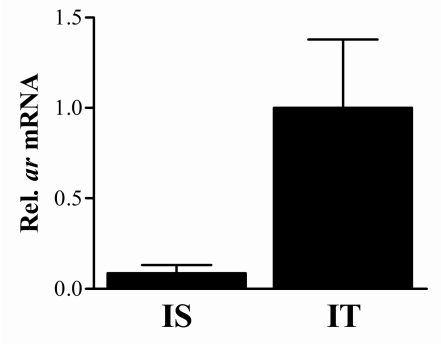

Figure 1. Relative zebrafish *ar* mRNA expression levels in two laser-dissected testis tissue fractions, interstitial (IS) and intratubular (IT). Values were determined from two experiments with duplicate measurements each (mean ± SEM). Values were normalized to *β-actin* mRNA levels, and expressed as fold of *ar* transcript amount in IT.

Experimental models to study endocrine regulation of adult zebrafish spermatogenesis

In search of *in vitro* models to study endocrine regulation of adult fish spermatogenesis, dissociated primary cell cultures and primary organ cultures were evaluated for their capacity to support spermatogenesis.

Certain differentiation steps of zebrafish spermatogenesis (*e.g.* [entry into] meiosis and spermiogenesis) are possible using co-cultures of germ cells and Sertoli cell-enriched feeder cell lines (Sakai 2002; Kurita and Sakai 2004). We obtained two Sertoli cell-derived testicular cell lines (ZtA6-3 and ZtA6-10) from Dr N. Sakai. These cell lines were generated by limiting dilution colony isolation from a Sertoli cell-enriched testicular primary cell culture derived from a testicular tumor (Sakai 2002; Kurita and Sakai 2004). Both cell lines had Sertoli cell characteristics but also distinct individual features, in terms of phagocytotic activity, gene expression and their capacity to support germ cell development in culture (Kurita and Sakai 2004). Similar to ZtA6-2 (Kurita and Sakai 2004), we

observed that ZtA6-3 cells (treated with mitomycin C to stop proliferative activity) seemed to have a higher capacity to adhere to and support large clusters of brightly fluorescent germ cells isolated from transgenic zebrafish, expressing EGPF under control of the *vasa* promoter (*vasa*::EGFP) (Krøvel and Olsen 2002), than ZtA6-10 did (unpublished observation); Vasa is highly expressed by spermatogonia, and expression decreases when cells enter meiosis. Interestingly, ZtA6-3 cells express *ar* but not *fshr* mRNA, while binding experiments indicated ZtA6-3 cells are able to bind radioactive T (but not 11-KT). However, the binding capacity was too low for a proper pharmacological analysis. Both androgens were unable to activate Ar-mediated reporter gene expression in ZtA6-3 cells transfected with an androgen-responsive promoter construct (De Waal P.P., Nijenhuis W.A., Schulz R.W., Bogerd J.; unpublished data). Since expression of *ar* mRNA by ZtA6-3 cells was quite low, we speculated that the amount of Ar protein was too low that binding of T was competed by another protein. However, obvious candidates, such as aromatase A (*cyp19a*) or *shbg* mRNA were not detected in ZtA6-3 cells (De Waal P.P., Schulz R.W., Bogerd J.; unpublished data). Furthermore, the expression of *cyp17a1* mRNA was detected in the ZtA6-3 cell extracts. Since Cyp17a1 is otherwise specifically expressed in zebrafish Leydig cells (**Chapter 4**), we concluded that ZtA6-3 was either contaminated with Leydig cells, or showed signs of de- or transdifferentiation. Finally, microarray analysis was attempted on Ar-transfected ZtA6-3 cells treated with methyl-testosterone (*i.e.* a potent synthetic androgen) or 11-KT in potent concentrations (100 nM) but no transcripts could be identified that were up- or down-regulated more than 2-fold (De Waal P.P., Bogerd J.; unpublished data). Moreover, to culture the ZtA6-3 cells (Sakai 2002; Kurita and Sakai 2004), large volumes of zebrafish extract had to be prepared to supplement the growth medium. Altogether, the evaluated Sertoli cell-enriched culture, although partially promising (*e.g.* as regards supporting germ cell development) raised too many issues to be considered as a valuable tool to study the mechanisms of endocrine regulation in Sertoli cells. Therefore, our attention was directed to testis organ culture as a possible tool.

Presently, complete spermatogenesis (*i.e.* from type A spermatogonia to spermatozoa) in culture has been reported for fish only (Miura *et al*. 1991). Moreover, certain steps in spermatogenesis (*e.g.* entry into meiosis) are possible in salmonid and amphibian testicular organ culture (Abe and Ji 1994; Amer *et al*. 2001). Possibly the less complex association of Sertoli cells and germ cells in anamniotes, as compared to amniotes, may allow spermatogenesis in medium-term culture. Therefore, fish would be par excellence the appropriate model to investigate adult spermatogenesis *ex vivo*. Until now testis organ culture systems

have been described using prepubertal testis, in which spermatogenesis was induced from testis containing type A spermatogonia and possibly type B spermatogonia (Miura *et al*. 1991; Abe and Ji 1994; Amer *et al*. 2001). In **Chapter 3** we describe the adult zebrafish testis organ culture system. The support of the complete spermatogenic process was possible in the presence of 11-KT. Morphological analysis and analysis of BrdU incorporation revealed an abundance of differentiating and differentiated germ cells (*i.e.* type B spermatogonia, spermatocytes, spermatids and spermatozoa) as well as the maintenance of the ability to produce new spermatogonia that proliferated and differentiated until spermatozoa. In the absence of 11-KT, on the other hand, the differentiated germ cell types (except spermatozoa) became depleted. In immature eel testis organ culture, 11-KT could induce spermatogenesis and support the remainder of the process (Miura *et al*. 1991). However, in contrast to our system, this required the additional presence of bovine insulin or human recombinant IGF1 as permissive factor (Miura *et al*. 1991; Nader *et al*. 1999). Another important feature of the organ culture system was the spontaneous and rapid (<48 h) down-regulation of the activity of the steroidogenic system. The down-regulation of the steroidogenic system can be considered an advantage, since it allows the evaluation of substances suspected to be stimulatory to spermatogenesis, without interference of endogenously produced steroids.

Next to primary culture approaches, we also evaluated possibilities for an *in vivo* system to study the endocrine regulation of spermatogenesis. An *in vivo* system allows assessment of ecotoxicological effects, and to observe the whole animal physiology. Furthermore, indirect effects on other components of the hypothalamus-pituitary-testis axis can be taken in consideration. An *in vivo* zebrafish androgen insufficiency model, induced by exposure to E_2, has been described in **Chapter 4**. Immunocytochemical analysis of germ cell proliferation and morphometric analysis revealed two prominent effects of the E_2 exposure: (i) the reduction, but not the abolishment, of proliferation of type A spermatogonia, and (ii) the inhibition of differentiation of type A into type B spermatogonia.

The mechanism behind the oestrogen-mediated inhibition of spermatogenesis involves the reduction of testicular androgen synthesis as a result of the reduced expression of steroidogenesis-related genes (*i.e. star*, *cyp17a1*). The observations that E_2 elicited no clear inhibitory effects on spermatogenesis and minor effects on the steroidogenic system in testis cultures *ex vivo* suggested that E_2 exerted its inhibitory effects on testicular physiology mainly via feedback mechanisms on the brain-pituitary system. We did not measure pituitary *fshb* or *lhb* subunit mRNA expression or Fsh or Lh plasma levels (no antibodies available) but

information from closely related species indicated E_2 exerts negative feedback on both Fsh and Lh synthesis and release (Billard and Peter 1977; Dickey and Swanson 1998; Kobayashi *et al*. 2001; Huggard-Nelson *et al*. 2002; Banerjee and Khan 2008).

Inhibitory E_2 effects on spermatogenesis, mediated by membrane-associated or nuclear oestrogen receptors, which are expressed in several piscine, testicular cell types (Miura *et al*. 1999; Bouma and Nagler 2001; Wu *et al*. 2001; Liu *et al*. 2009), were not evident as was shown by E_2 exposure of zebrafish testis *ex vivo* in the presence of recombinant piscine Igf1 (to maintain spermatogenesis in a steroid-independent manner; see **Chapter 5**). Although, the effects on the steroidogenic system were clear, the contribution of possibly reduced Fsh plasma levels to the phenotype of this model has to be considered. Therefore, we were interested whether androgen treatment (in the presence of E_2) could reverse the disruption of spermatogenesis in zebrafish exposed to E_2 for 3 weeks. We chose 11-ketoandrostenedione (OA, 100 nM) for this androgen treatment, because OA is converted to 11-KT by Hsd17, which is expressed in several organs in zebrafish (Mindnich *et al*. 2005), and OA treatments led to increased 11-KT plasma levels and induced spermatogenesis in other fish species (Cavaco *et al*. 1998; Antonopoulou *et al*. 1999). After 2 weeks of OA treatment, testis weight and GSI increased significantly (Fig 2A, 2B). Morphological analysis showed depletion of differentiating germ cells (*i.e.* type B spermatogonia, spermatocytes and spermatids) in testes of zebrafish exposed to E_2 for 5 weeks, whereas additional exposure to OA for the final 2 weeks induced the re-appearance of all differentiating germ cell types in abundant numbers (Fig 2C, 2D). This experiment indicated that 11 oxygenated androgens (OA and/or 11-KT) restored spermatogenesis in E_2-exposed zebrafish. However, detailed morphometric analysis and quantification of germ cell proliferation analysis has to show the exact stages of germ cell development affected by androgen exposure. Feedback mechanisms of non-aromatisable, 11-oxygenated androgens on Fsh pituitary and plasma content were reported to be mainly negative (Antonopoulou *et al*. 1999; Schulz *et al*. 2008), so that Fsh levels were probably not restored. However, the role(s) of Fsh – independent of its steroidogenic function – should preferentially be studied in a more direct manner (see below).

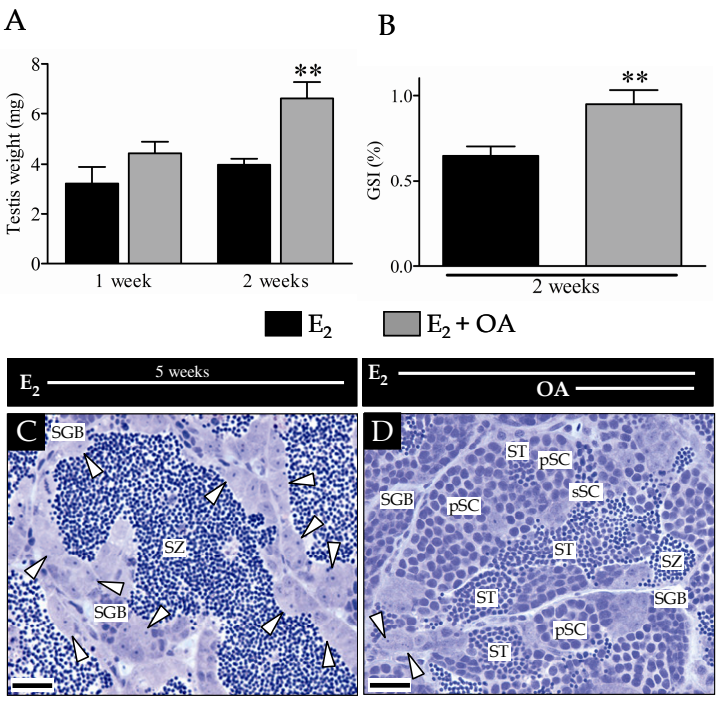

Figure 2. Androgen treatment reverses inhibitory effects of E_2 pretreatment on adult zebrafish spermatogenesis. (A) Testis weights (expressed in mg; mean ± SE) and (B) GSI (% of body weight; mean ± SE) of E_2-exposed zebrafish maintained in the absence or the presence of 100 nM OA for 1 week (n=6-8 fish per treatment) or 2 weeks (n=8-9 fish per treatment). Bars marked with ** ($P<0.01$) are significantly different from control. Zebrafish testis sections from (C) exposed fish to E_2 only for 5 weeks, or (D) from fish also exposed to OA during the final 2 weeks. Arrowheads, type A spermatogonia; SGB, type B spermatogonia; pSC, primary spermatocytes; sSC, secondary spermatocytes; ST, spermatids; SZ, spermatozoa;. Scale bars in (C) and (D) = 25 µm.

Androgen-sensitive phases during adult piscine spermatogenesis

The experimental *in vitro* and *in vivo* models described in **Chapters 3** & **4** served as a means to identify androgen-dependent phases of the spermatogenic process.

In adult zebrafish testis organ culture, 11-KT maintained all stages of spermatogenesis and the ability of germ cells to proliferate and differentiate (**Chapter 3**). Moreover, the BrdU exposure experiment described in **Chapter 5** showed that 11-KT increased the mitotic index of type A spermatogonia and number of cysts containing proliferating type B spermatogonia. This indicates that 11-KT stimulated the commitment of new spermatogonia to enter the differentiation pathway throughout the culture period. However, also under basal culture conditions the proliferation of type A spermatogonia was not completely abolished suggesting that other factors play a role as well. In comparison, in

juvenile eel and huchen (*Hucho perryi*) testis organ cultures, 11-KT (albeit in the presence of insulin or IGF1) stimulated spermatogonial proliferation and differentiation (*i.e.* increased presence of proliferating late type B spermatogonia), and allowed entrance into the meiotic phase (Miura *et al*. 1991; Amer *et al*. 2001). In addition, 11-KT supported the completion of meiosis and spermiogenesis to supply fully differentiated spermatozoa in juvenile eel testis culture (Miura *et al*. 1991).

In vivo, androgen insufficiency in zebrafish spermatogenesis resulted in a depletion of differentiating germ cells (i.e. type B spermatogonia, spermatocytes and spermatids) due to reduced proliferation of type A spermatogonia and differentiation into type B spermatogonia (**Chapter 4**). Since germ cell depletion was increasingly prominent with progressive differentiation, this suggested that (post)meiotic phases could have been affected as well. However, to investigate specific androgen support to these phases of spermatogenesis *in vivo* other experiments should be performed. Androgens could restore spermatogenesis in zebrafish suffering from androgen insufficiency (see above). In comparison, in juvenile catfish (10-11 weeks old), which exhibit type A and some type B spermatogonia only, 11-oxygenated androgens stimulated spermatogonial proliferation and supported germ cell passage through meiosis *in vivo* (Cavaco *et al*. 1998).

Differences exist in the sites of androgen action during spermatogenesis between the most frequently studied mammalian species, rodents and primates. Androgen depletion or blockage in androgen signalling in rodents affected more prominently the (post)meiotic stages of spermatogenesis, whereas spermatogonial proliferation remained relatively unaffected (O'Donnell *et al*. 1994; El Shennawy *et al*. 1998; França *et al*. 1998; De Gendt *et al*. 2004; McLachlan *et al*. 2002b; Sharpe 2005). As such, rodents do not serve as an appropriate model to investigate androgen actions on spermatogonial proliferation and differentiation. On the other hand, androgens are effective in preventing germ cell apoptosis (*e.g.* intermediate spermatogonia), especially in synergism with FSH (El Shennawy *et al*. 1998). A prominent androgen-sensitive phase of rodent spermatogenesis is the attachment of round spermatids to Sertoli cells (O'Donnell *et al*. 1994), through a specialized junction complex, called the ectoplasmic specialization. However, such a specialized junction has not been reported in teleosts (Batlouni *et al*. 2009).

On the other hand, in primates the spermatogonial compartment is more prominently affected after gonadotropin (*i.e.* FSH and LH) withdrawal in comparison to rodents (Marshall *et al*. 2005; McLachlan *et al*. 2002a). Particularly, A_{pale} and B type spermatogonia are reported to be hormone-sensitive targets; differentiation of A_{pale} into B type spermatogonia depends on T and/or FSH action

(Marshall *et al*. 2005). This shows some degree of resemblance to the hormonal dependency of type A spermatogonia to proliferate and differentiate into type B spermatogonia in fish spermatogenesis. Similar to fish, specific contribution of androgens and FSH still remain to be elucidated in primate spermatogenesis. In contrast, germ cells passing through meiosis and spermiogenesis were largely unaffected after gonadotropin withdrawal in primates (McLachlan *et al*. 2002a).

In summary, our experimental models indicate that androgens support the three main phases (*i.e.* mitotic, meiotic and spermiogenic) of adult fish spermatogenesis. High expression of *ar* mRNA in Sertoli cells surrounding spermatogonia (**Chapter 2**; (Almeida *et al*. 2009), stimulation of proliferation of type A and type B spermatogonia (**Chapter 5**), and the marked effect on spermatogonial proliferation and differentiation in an androgen-depletion model (**Chapter 4**) may suggest a high sensitivity of the spermatogonial phase to androgen regulation. In addition, initial small rises in 11-KT plasma levels were correlated with the stimulation of spermatogonial proliferation and entry into meiosis, whereas 11-KT plasma levels required to rise even further to allow germ cells to pass through meiosis and spermiogenesis, in the annual cycle of male Chinook salmon (*Oncorhynchus tschawytscha*) (Campbell *et al*. 2003).

Endocrine regulation of piscine Sertoli cell-mediated paracrine action

In zebrafish, *ar* is highly expressed by Sertoli cells surrounding type A spermatogonia (**Chapter 2**). Studies in eel have shown high Fshr immuno-reactivity in Sertoli cells surrounding type A and B spermatogonia (Ohta *et al*. 2007). Fsh and 11-KT levels rise in plasma at the start of the spermatogonial proliferation in Chinook salmon (*Oncorhynchus tshawytscha*) (Campbell *et al*. 2003). In juvenile Japanese eel, Sertoli cells mediate 11-KT action on spermatogonia by reducing the secretion of an inhibitory factor (Miura *et al*. 2002) or promoting the expression of a stimulatory factor (Miura *et al*. 1995), thereby providing the suitable microenvironment for spermatogonial differentiation. Respective information in other fish species, or information on androgen-independent Fsh action on spermatogonesis in fish mediated by Sertoli cell-secreted factors, is largely missing.

In **Chapter 5**, we investigated the testicular expression pattern, endocrine sensitivity, and the biological activity on zebrafish spermatogenesis of an interesting family of growth factors previously shown to have mitogenic effects on spermatogonia among vertebrates (Dubois and Callard 1993; Li *et al*. 2008; Loir

1994, 1999; Loir and Le Gac 1994; Söder *et al*. 1992): the insulin-like growth factor (IGF) family.

We found that all IGFs and their type 1 IGF receptors are expressed in the somatic compartment but others have reported IGF protein expression in piscine germ cells as well (Perrot *et al*. 2000; Reinecke *et al*. 1997). Our *in situ* hybridisation analysis revealed particularly high *igf3* mRNA levels in those Sertoli cells, associated with type A spermatogonia. The *igf3* gene has been discovered recently in tilapia (*Oreochromis niloticus*), zebrafish and medaka (*Oryzias latipes*); the *igf3* sequences are distinct from mammalian and amphibian IGF sequences, and *igf3* mRNA expression is restricted to the gonad in both sexes (Wang *et al*. 2008).

Our studies revealed a differential hormonal effect among the individual IGFs, By evaluating our androgen-insufficiency model (**Chapter 4**), *in vivo* gonadotropin-injected zebrafish, and testis organ cultures incubated with gonadotropin/11-KT (**Chapter 3**), we demonstrated androgenic stimulation of *igf3* mRNA levels *in vivo* and *ex vivo*. Co-incubations of testis organ cultures with a 3βHSD inhibitor (trilostane) showed that *igf3* was regulated by Fsh, independent of biologically active steroids. However, the effect of Fsh on germ cell development in adult fish remains to be investigated. In juvenile Japanese eel, the Fsh-induced stimulus on spermatogonial proliferation disappeared in the presence of trilostane (Ohta *et al*. 2007). Co-incubation of zebrafish testis organ cultures with Fsh and trilostane will serve as a powerful means to investigate this question.

Based on the strong expression in Sertoli cells contacting type A spermatogonia, and on the regulation by androgens and Fsh, we hypothesise that *igf3* is a factor secreted by Sertoli cells providing a suitable microenvironment for proliferation and/or differentiation of spermatogonia. The next step would be to evaluate the biological activity of recombinant zebrafish Igf3 on spermatogenesis. However, since the protein is not available yet, the biological activity of recombinant gilthead seabream Igf1 was investigated on testis organ culture. As determined by morphometric analysis, at concentrations of 25 ng/ml Igf1 had a clear stimulatory effect on the proliferation and differentiation of spermatogonia and their entry into meiosis and spermiogenesis. Since Igf1 was reported to modulate steroid production we measured 11-KT release in testis organ culture after different conditions but observed no increase in 11-KT production or prevention of the down-regulation of the steroidogenic system. This indicated that Igf1 alone (without stimulating androgen production) was able to maintain the mentioned steps in germ cell development *ex vivo*. In juvenile eel, Igf1 has a permissive role in 11-KT induced spermatogenesis, but is not able to induce spermatogenesis alone (Nader *et al*. 1999). Furthermore, Igf1 enhanced 11-KT

stimulated zebrafish spermatogenesis *ex vivo*. The combined presence of both substances resulted in a strong differentiation signal. A possible explanation for this observation may be related to the Igf-mediated inactivation of a co-repressor of Ar-mediated gene transcription (Fan *et al*. 2007).

Final remarks

The present thesis obtained more knowledge on the sites of action and the mechanisms of endocrine control of adult fish spermatogenesis. The possibility to study the maintenance of fish spermatogenesis in culture provides a great asset to study effects of the individual pituitary gonadotropins, growth factors, (sex steroids), directly on testicular physiology. Importantly, zebrafish have proven to be an interesting model to study mechanisms of endocrine control of the mitotic phase, and can serve as translational window to study the endocrine control of conserved Sertoli cell-secreted factors influential to germ cell development. Illustrative of the conservation was the demonstration of endocrine control of a fish representative of the IGF family, known to act as mitogen on spermatogonia across vertebrates. Although some aspects of Fsh-mediated mechanisms independent of androgen production were revealed, future work has to concentrate on the identification of the contribution of Fsh control on adult fish spermatogenesis independent of steroid synthesis.

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Samenvatting

In de testis vindt een complex en strak georganiseerd proces plaats waarbij spermatogoniale stamcellen zich ontwikkelen tot spermatozoa: de spermatogenese. In hogere vertebraten wordt de spermatogenese endocrien gereguleerd door voornamelijk twee hormonen: hypofysair follikel stimulerend hormoon (FSH), en androgenen, waarvan de testiculaire productie en afgifte gereguleerd wordt door hypofysair luteïniserend hormoon. Anders dan bij zoogdieren, dient men bij vissen rekening te houden met het potente effect van Fsh op de steroïdsynthese. Dit suggereert dat Fsh activiteit op de spermatogenese bij vissen deels gemedieerd wordt door androgenen. Het doel van dit proefschrift was het ophelderen van de mechanismen hoe Fsh en, in het bijzonder, androgenen de adulte spermatogenese reguleren, met nadruk op de spermatogoniale fase. Als proefdiermodel werd de zebravis gekozen.

Eerst werd de route van androgeen synthese beschreven en werd de cruciale mediator van androgene bioactiviteit, de androgeen receptor (Ar), gekarakteriseerd. 11-ketotestosteron (11-KT) werd geïdentificeerd als het belangrijkste eindproduct van de androgeen biosynthese route in zebravis testis. Testosteron, een prominent androgeen in het serum van verwante vissensoorten, en 11-KT binden en activeren de zebravis Ar in lage (nanomolaire), en dus physiologisch relevante, concentraties.

Vervolgens werden proefmodellen ontwikkeld waaruit bleek dat de spermatogenese in vissen afhankelijk is van androgeen regulatie, en dat vooral de spermatogoniale fase daarvoor gevoelig is. In een *ex vivo* orgaankweekmodel voor volwassen zebravis testis werd de gehele spermatogenese ondersteund in de aanwezigheid van 11-KT, terwijl in de afwezigheid van 11-KT de spermatogenese tot stilstand kwam, waarschijnlijk als gevolg van een spontane en snelle afname van de activiteit van de steroïdsynthesemachinerie onder deze kweekconditie.

Uit een *in vivo* model, welke androgeeninsufficiënt gemaakt werd door oestrogeenbehandeling, bleek een opvallende inhibitie van de proliferatie en differentiatie van spermatogoniën. Dit *in vivo* model kwam tot stand door negatieve oestrogene feedback op de hersen-hypofyse-as. Dit leidde tot een afname van de expressie van factoren betrokken bij de androgeen synthese en uiteindelijk tot een gereduceerde androgeen productie.

Sertoli cellen brengen, in tegenstelling tot kiemcellen, de functionele receptoren voor zowel Fsh en androgenen tot expressie. Een interessante vondst, daarom, was dat Sertoli cellen, die A spermatogoniën omringen, hoge *ar* expressie vertonen. Één van de manieren hoe Sertoli cellen de activiteit van Fsh of

androgenen mediëren is door de secretie van paracriene factoren te reguleren. Deze op hun beurt beïnvloeden de proliferatie en differentiatie van spermatogoniën. Van een gonadespecifieke representant, die alleen is gevonden in vissen (nl. *igf3*), van een groep paracriene factoren, de *insulin-like growth factors*, werd een hoge expressie bepaald in zebravis Sertoli cellen, die A spermatogoniën omringen. Verder werd de mRNA expressie van *igf3* positief gemoduleerd door zowel androgenen als Fsh, en voor Fsh onafhankelijk van zijn invloed op de steroïdsynthese. Uit onderzoek naar het effect van een commercieel verkrijgbaar recombinant vissen Igf eiwit (*i.e.* Igf1) op de zebravis spermatogenese *in vitro,* bleek dat de proliferatie van spermatogoniën en hun overgang naar de meiose gestimuleerd werden.

Dit proefschrift heeft een bijdrage geleverd aan de kennis van de plaats van werking, en de mechanismen van hormonale regulatie van spermatogenese in adulte vissen. Er werden nieuwe proefmodellen ontwikkeld om de hormonale regulatie van de spermatogenese te onderzoeken. De zebravis is zeker een boeiend en uitermate geschikt proefdiermodel voor onderzoek naar de hormonale regulatie van spermatogenese.

Summary

In the testis a highly complex and tightly orchestrated process takes place, during which spermatogonial stem cells develop into spermatozoa. This process is known as spermatogenesis. In higher vertebrates, spermatogenesis is under the endocrine control of mainly two hormones, the pituitary-derived follicle-stimulating hormone (FSH) and androgens; the testicular production and secretion of the latter are controlled by the pituitary-derived luteinizing hormone (LH). In fish, different from mammals, also the strong steroidogenic potency of Fsh must be taken into account, suggesting that part of Fsh action on fish spermatogenesis is mediated by androgens. This thesis aimed at elucidating the mechanisms, by which Fsh and especially androgens regulate adult spermatogenesis, with an emphasis on the spermatogonial phase. Zebrafish was chosen as the experimental animal model.

First, the pathway of androgen synthesis was described and the crucial mediator of androgen bioactivity, the androgen receptor (Ar) was characterised. These studies identified 11-ketotestosterone (11-KT) as the main end product of the androgen biosynthesis in zebrafish testis. Together with testosterone, a prominent androgen in the serum of closely related fish species, 11-KT binds to and activates the zebrafish Ar at low nanomolar, and thus physiologically relevant, concentrations.

Subsequently, experimental models were developed with which it could be revealed that adult fish spermatogenesis is dependent on androgen regulation, and that the spermatogonial phase is particularly sensitive. In an *ex vivo* organ culture system for adult zebrafish testis, complete spermatogenesis was supported in the presence of 11-KT while in the absence of 11-KT, spermatogenesis became arrested, probably related to the spontaneous and rapid decrease of activity of the steroidogenic system under basal culture conditions.

An *in vivo* model based on an oestrogen-induced androgen insufficiency served to reveal a marked inhibition of spermatogonial proliferation and differentiation. This *in vivo* model exploited the negative oestrogenic feedback on the brain-pituitary system, causing a reduction of expression of factors otherwise responsible for androgen synthesis, ultimately leading to reduced testicular androgen production.

Sertoli cells express functional receptors for both FSH and androgens, in contrast to germ cells. It was therefore interesting to find that zebrafish Sertoli cells contacting type A spermatogonia express high levels of *ar* transcript. One of the ways Sertoli cells mediate androgen or Fsh action by modulating the secretion of paracrine factors, which in turn influence spermatogonial proliferation and/or differentiation. We showed that the gonad-specific fish representative of such a group of paracrine factors, the insulin-like growth factors, in particular *igf3*, was highly expressed by zebrafish Sertoli cells contacting type A spermatogonia. Furthermore, *igf3* mRNA expression was positively modulated by both androgens as well as Fsh, for the latter independent of its steroidogenic potential. Studies on the action of a commercially available recombinant fish Igf protein (*i.e.* Igf1) on zebrafish spermatogenesis *in vitro* revealed a stimulatory effect on the proliferation of spermatogonia and their entrance into meiosis.

The present thesis expanded the knowledge on the sites of action and the mechanisms of endocrine control of adult fish spermatogenesis. In addition, novel experimental models to investigate the endocrine control of adult spermatogenesis were developed. Clearly, zebrafish is an exciting and excellently suited animal model also for investigations on the endocrine control of spermatogenesis.

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Curriculum Vitae

Paulus Petrus de Waal werd geboren op 7 maart 1980 in Bergen op Zoom. Na het behalen van het VWO diploma aan het Mollerlyceum te Bergen op Zoom in 1998, studeerde hij Biomedische Wetenschappen aan de Universiteit Leiden. Zijn doctoraal behaalde hij in november 2003 na een afstudeerstage bij het Laboratorium voor Experimentele Patho-Oncologie van het Josephine Nefkens Instituut (Erasmus MC) te Rotterdam, waar hij mogelijke tumor suppressor genen in testiculaire kiemceltumoren onderzocht onder leiding van dr. A.M. Zeeman en Prof. dr. L.H.J. Looijenga.

In januari 2005 trad hij in dienst als assistent in opleiding bij de leerstoelgroep Endocrinologie (huidige Endocrinologie en Metabolisme) aan het Department Biologie van de Universiteit Utrecht, waar hij onder leiding van Prof. dr. D.G. de Rooij, dr. J. Bogerd en dr. R.W. Schulz onderzoek verrichtte aan het project, waarvan de resultaten beschreven staan in dit proefschrift.

Sinds juni 2009 is hij werkzaam als Moleculair Bioloog bij de afdeling Research & Development van de Koninklijke Nedalco te Bergen op Zoom.

List of Publications

P.P. de Waal, M.C. Leal, Á. García-López, S. Liarte, H. de Jonge, N. Hinfray, F. Brion, R.W. Schulz, J. Bogerd 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

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