
Endocrine Regulation of Spermatogenesis in Teleost Fish

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Abstract

Mitotic proliferation of spermatogonia, meiosis of spermatocytes, and the restructuring of round spermatids into flagellated spermatozoa (spermiogenesis), are hallmark events of spermatogenesis. The process is fuelled by stem cells that either self-renew or produce spermatogonia committed to further development. Germ cells always require the close contact with Sertoli cells. These somatic cells, but not germ cells, express receptors for androgens and follicle-stimulating hormone (FSH), thus functioning as interface between hormones and the developing germ cells. In Japanese eel, for example, gonadotropin or 11-ketotestosterone (11-KT; a typical androgen in fish) stimulation induces complete spermatogenesis *ex vivo*, and spermatogonial proliferation is in part mediated by Sertoli cell-derived activin. In fish LH, but also FSH, stimulates steroidogenesis. Since FSH but not LH circulates in sexually immature salmonids, increased FSH signalling may be sufficient to initiate spermatogenesis by activating both Sertoli cell functions and 11-KT production. Fish testes also produce testosterone (T), which exerts negative feedback effects on FSH-dependent signalling and on steroidogenesis. The balance between T and 11-KT production and plasma levels therefore is important. Besides inducing spermatogonial proliferation, 11-KT also induces meiosis and complete spermiogenesis in tissue culture. However, it is not known yet by what mechanisms 11-KT stimulates the passage of germ cells through these more advanced stages of development.

Key words: spermatogenesis, androgens, follicle-stimulating hormone, Sertoli cell, growth factors, fish, puberty.

Invited Mini-review

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Introduction and Bird's-Eye-View on Spermatogenesis

The cellular basis of male reproduction is the spermatozoon, which develops from stem cells in a suite of proliferation and differentiation steps known as spermatogenesis. For this development, germ cells require the environment created by Sertoli cells. These somatic cells envelope germ cells, thus forming Sertoli/germ cell units. Besides paracrine communication within these units, the brain-pituitary-gonad (BPG) axis has evolved as hormonal master control system over spermatogenesis and reproduction in general, adjusting the activity of the reproductive system according to the environmental conditions on the one hand, and to the developmental and physiological state of the individual on the other hand.

When stem cells proliferate, they provide either new stem cells (single primary, or A_s spermatogonium), or spermatogonia that are committed to differentiation. There is no formal proof yet that (a portion of) the A_s spermatogonia indeed have stem cell capacities in fish. However, repetitive waves of spermatogenesis are observed in many species, suggesting that stem cells are present also in fish testis. The balance between self-renewal and differentiation seems to be regulated (see below). The number of spermatogonia increases rapidly during a species-specific number of mitotic cell cycles. In the guppy, for example, spermatogonia go through 14 mitotic cell cycles (Billard, 1969^a), and 10 have been reported in the Japanese eel (Miura *et al.*, 1991^a). In some species, not a specific number but rather a range of mitotic cell cycles was observed, for example, 5 or 6 in zebrafish (Ewing, 1972). There is no consensus concerning the terminology to address the different spermatogonial generations in fish. In mammals, early generations of slowly dividing spermatogonia are referred to as undifferentiated A spermatogonia. They differentiate via retinoic acid signalling to enter strictly timed, rapid mitotic cell cycles that produce the successive generations of differentiating spermatogonia, the final generation is referred to as B spermatogonia (de Rooij & Russell, 2000). It is proposed to adopt this terminology for fish spermatogenesis, since distinguishing undifferentiated, slowly dividing, early spermatogonia from rapidly dividing, differentiating A spermatogonia seems justified also in fish. In the Japanese eel, for example, human chorionic gonadotropin (hCG) treatment stimulates proliferation of early spermatogonia in immature males; however, this ceased after the 4th or 5th (of 10 in total) mitosis in some fish ("non-responders"), suggesting that a regulatory checkpoint separates early (undifferentiated) from late (differentiating) spermatogonia, and once this checkpoint is passed, entry into meiosis with its two specialised cell cycles seems possible (Miura *et al.*, 1997). During the first one, DNA is duplicated for the last time and the genetic information is recombined in the primary spermatocytes. They divide to form short-lived secondary spermatocytes, which divide again quickly without DNA duplication, giving rise to haploid spermatids. These small, round cells undergo spermiogenesis, a cellular restructuring to flagellated spermatozoa without further proliferation. During

spermiogenesis germ cells lose 80-90% of the cellular and nuclear volume (Sprando *et al.*, 1988) by chromatin condensation, shedding of nucleoplasm into the cytoplasm, and by extrusion of cellular material, which is then phagocytised by Sertoli cells. An acrosome does not develop in most fishes, since the postovulatory oocyte is not surrounded by granulosa cells and has a micropyle to allow sperm entry (Patiño & Sullivan, 2003). After completion of spermiogenesis, the cyst wall opens to release the spermatozoa (spermiation). The total duration of the process is determined by the germ cells' genome (França *et al.*, 1998), is strictly timed in mammals, and perhaps other warm-blooded vertebrates as well, and takes, for example, 59 or 39 days in rat or mice, respectively (Russell *et al.*, 1990). In cold-blooded vertebrates, temperature influences the timing of spermatogenesis but the duration is predictable also in fish at a given temperature (Billard 1968; Vilela *et al.*, in press).

The Sertoli – Germ Cell Relation in Cystic Spermatogenesis

Germ cell survival and development strictly depends on their intimate association with Sertoli cells, thus forming Sertoli/germ cell units. In fish, such a unit forms when Sertoli cells envelope a single early spermatogonium; the Sertoli cells, but not the germ cells, are in contact with the basement membrane that separates germinal and interstitial compartment of the testis (Loir *et al.*, 1995). Such a unit is known as spermatogenic cyst or spermatocyst. Collectively, the spermatocysts form the germinal epithelium of the teleost testis.

There are two different types of testicular architecture in teleosts (Grier & Taylor, 1998; Loir *et al.*, 1995). In the anastomosing type, stem cells are distributed throughout the testis (“unrestricted”), spermatocysts do not move during maturation, and anastomoses are observed between the tubules; this type is found in most fishes. In higher perciform and in atheriniform fish, however, the tubules end blind in the ventro-lateral periphery of the testis (and can therefore be referred to as lobules). In this type, the spermatogonial stem cells are restricted to the tips of the lobules, and the spermatocysts are displaced towards the dorsally located efferent duct system during maturation.

Germ cell number and spermatocyst volume increase rapidly during the mitotic phase. The number of Sertoli cells associated with a developing germ cell clone is increasing as well in species with a restricted distribution of stem cells (Billard, 1969^a; Vilela *et al.*, in press), while respective information is not available from species showing the anastomosing testis type. Since all germ cells within one spermatocyst are clonal descendents of one stem cell, a given Sertoli cell contacts germ cells that are all in the same stage of development. In amniote vertebrates, on the other hand, a given Sertoli cell concomitantly contacts members of several germ cell clones (Russell *et al.*, 1990). This suggests that at any given point in time Sertoli cell functions are comparatively less complex in fish than in mammals, while overall, similar functions are fulfilled. This makes fish an attractive experimental model for studies on Sertoli cell functions.

Endocrine Regulation of Spermatogenesis – Pituitary Hormones and Sex Steroids

Which steps in spermatogenesis might be responsive to hormones? The number of germ cell divisions is determined genetically. In some species (see earlier discussion), a range rather than a fixed number of cell cycles is observed, but there are no experimental data available in fish in this context. Also an effect of hormones on the timing of most of the developmental steps in spermatogenesis is unlikely, viz. that the different developmental stages are exposed to a similar endocrine environment. As regards meiosis and spermiogenesis, hypophysectomy experiments indicated (see below) that hormone-dependent checkpoints exist, between which developmental processes seem to occur according to genetically fixed programmes. Other possible targets of hormone action are i) the stem cell activity, *i.e.* the balance between self-renewal and the production of differentiating germ cells, which determines if, and how many, cells enter the process of spermatogenesis; ii) the recruitment of early spermatogonia into the rapid proliferation period and their entrance into meiosis; (iii) the loss of germ cells, in particular spermatocytes and spermatids, via apoptosis; and finally (iv) Sertoli cell proliferation and differentiation, a process determining the germ cell production capacity.

Pituitary Gonadotropins

In sexually immature eel, the testis contains single spermatogonia and small clones of undifferentiated spermatogonia. Following hypophysectomy, the small spermatocysts disintegrate, leaving single spermatogonia as the only germ cell type (Khan *et al.*, 1986). This suggests that the balance between stem cell renewal and production of spermatogonia is shifted towards the former in the absence of the pituitary.

Hypophysectomy blocked spermatogonial differentiation at the 7th (of 14) mitotic division in the guppy (Billard 1969^b); also reduced numbers of early spermatogonia were observed. In addition, B spermatogonia failed to undergo the final mitosis, so that primary spermatocytes were not formed. Primary spermatocytes were arrested at the end of the prophase of the 1st meiotic cell cycle, and also secondary spermatocytes failed to enter the 2nd meiotic M-phase. Early spermatids commenced spermiogenesis but degenerated before its completion, indicating that also postmeiotic development requires pituitary regulation.

Germ cell loss is not only observed under experimental conditions, but seems to be a regulatory mechanism of normal spermatogenesis. For example, about one third of the germ cells are lost under normal (control) conditions during spermatogenesis in the guppy (Billard 1969^a) or in tilapia (Vilela *et al.*, in press). Germ cell loss is common also in mammals, where it is mediated via apoptosis (Sinha-Hikim *et al.*, 1997). Germ cell apoptosis has been described in the piscine testis as well (*e.g.* Uchida *et al.*, 2002), but the regulatory background of this event is unexplored.

The bioactive compounds in the pituitary are the gonadotropins, LH and FSH.

LH stimulates androgen production in Leydig cells via activation of the LH receptor (LH-R). Leydig cells are located between the seminiferous tubules in the interstitial area of the testis, from where androgens reach the seminiferous tubules, mediating the biological activity of LH. Within the tubules, androgens are required to drive spermatogenesis via the Sertoli cells that – in contrast to germ cells – express androgen receptors. The second gonadotropic hormone, FSH, directly stimulates the FSH receptor (FSH-R) expressing Sertoli cells, which in turn support germ cell survival and development.

This brief summary of gonadotropin functions is based on information from mammalian models. How is the situation in fish? When highly purified teleost gonadotropins became available (*e.g.* Kawachi *et al.*, 1989), both gonadotropins showed prominent steroidogenic potency, in particular in fish at the onset of spermatogenesis and during rapid testicular growth (Planas & Swanson, 1995). Ligand binding studies provided evidence for the presence of two gonadotropin receptors in salmon (Yan *et al.*, 1992), and moreover suggested that the FSH-R-like receptor, although having a preference for FSH, also shows affinity to LH (Miwa *et al.*, 1994). The apparently limited selectivity in hormone binding of piscine FSH-Rs has been confirmed in recent molecular studies (Bogerd *et al.*, 2001; Kumar *et al.*, 2001). The testicular cell types expressing FSH-R and LH-R still have to be identified unequivocally. It appears reasonable to assume that Leydig cells express LH-R and Sertoli cells express FSH-R, but it is not clear yet how FSH stimulates Leydig cell androgen production in fish. FSH-triggered Sertoli cell growth factor release to modulate Leydig cell androgen production, as described by Lejeune *et al.* (1996), might be one explanation.

While the main role of LH is to stimulate androgen production, the role of FSH seems more complex. Indeed, FSH has been referred to as pleiotropic hormone in context with its multiple activities in mammals (*e.g.* Arey *et al.*, 1997). In male fish, however, there is little information on the physiological roles(s) of FSH, besides the steroidogenic activity discussed above. The difficulty in obtaining highly purified FSH is one limiting factor. Moreover, FSH dosage systems with a high specificity and sensitivity are available for only a few species.

In the mammalian testis, only Sertoli cells express the FSH-R gene (Themmen *et al.*, 1994). These cells provide critical support to the germ cells' survival and development. Since a Sertoli cell has a finite capacity to support germ cells, the Sertoli cell number limits the sperm production capacity of the testis. Therefore, Sertoli cell proliferation is an important regulatory target. Delaying Sertoli cell maturation prolongs their proliferation period, hence resulting in larger testes with a higher spermatogenic output in rodents (Cooke *et al.*, 1994) and in tilapia (Matta *et al.*, 2002). Interfering with FSH signalling compromised Sertoli cell proliferation in mice and resulted in small testes (30% of control; Kumar *et al.*, 1997). One possible field of action of FSH in the fish testis therefore is the regulation of Sertoli cell proliferation, which may be of particular relevance in species with considerable, seasonal changes in testis weight. Besides effects on Sertoli cell

proliferation, FSH stimulates Sertoli cell differentiation in conjunction with androgens and thyroid hormones (Plant & Marshall, 2001). Only differentiated Sertoli cells can support meiotic and postmeiotic stages (Krishnamurthy *et al.*, 2001), so that delaying Sertoli cell maturation also delays meiosis in tilapia (Matta *et al.*, 2002).

The recent cloning of FSH-Rs from different fishes as well as the cloning of FSH β -subunits from several species provides tools to increase our understanding of the role of FSH in fish spermatogenesis. An important aspect of FSH action is to modulate the release of growth factors by Sertoli cells. The growth factors then function in an auto- or paracrine manner to regulate Sertoli and germ cell proliferation and differentiation. For example, members of the transforming growth factor β (TGF β ; *e.g.* Miura *et al.*, 1995; 2002), of the insulin-like growth factor (IGF; LeGac *et al.*, 1996), or of the fibroblast growth factor (FGF; Watanabe & Onitake, 1995) families of growth factors, play important roles, in particular with respect to regulating spermatogonial proliferation.

Steroid Hormones

Sex steroids are required for spermatogenesis. Both, FSH and LH stimulate steroid production in fish (Planas & Swanson, 1995). In tetrapod vertebrates, the biologically most active androgens are testosterone (T) and its 5α -reduced derivative dihydrotestosterone (DHT). Fish testes mainly produce 11-oxygenated androgens, such as 11-ketotestosterone (11-KT; Borg, 1994); while T is produced also, DHT is not found in fish. In some fish, the testes produce 11-oxygenated precursors of 11-KT, which are then converted to 11-KT by enzymes in tissues outside the testis (Cavaco *et al.*, 1997; Mayer *et al.*, 1990). Androgens modulate their own production which, in mammals, is mediated via androgen receptor (AR) dependent repression of steroidogenic enzyme expression (Payne & Youngblood, 1995). In juvenile male African catfish, treatment with 11-KT or T inhibited testicular C_{17-20} lyase activity and resulted in a loss of mitochondria and a reduced Leydig cell size (Cavaco *et al.*, 1999). These data suggest that androgen-mediated regulation of androgen production may constitute a feedback loop to prevent excessive androgen production.

Androgens exert biological activity by binding to the AR, nuclear receptors that function as ligand-activated transcription factor. The AR is expressed in Sertoli cells and in other testicular somatic cells, while germ cells do not express known AR forms (Ikeuchi *et al.*, 2001). Therefore testicular somatic cells represent the main interface between the developing germ cells and the endocrine system. In fish, two different types of androgen receptors have been identified by biochemical (Sperry & Thomas, 1999) and molecular approaches (Takeo & Yamashita, 1999; Ikeuchi *et al.*, 2001). One of these receptors binds different types of androgens, including T and 11-KT (Sperry & Thomas, 1999; Takeo & Yamashita, 2000) while the other receptor type has a preference for T (Sperry & Thomas, 1999). No information is available yet with regard to the regulation of testicular AR expression in fish.

What are androgens required for in spermatogenesis and how do they exert these effects? It is reasonable to assume that the effects observed following hypophysectomy (see earlier discussion) are related, in part, to the androgen shortage resulting from the absence of gonadotropic hormones. A mutation of the mouse AR is associated with infertility (Lyon *et al.*, 1975). Spermatogonial proliferation still takes place in these animals, while meiosis becomes arrested in the pachytene stage. When spermatogonial stem cells of AR mutant mice are implanted into a wild-type testis, the germ cells complete spermatogenesis, demonstrating that AR expression in the somatic compartment of the testis is sufficient for spermatogenesis (Johnston *et al.*, 2001). Therefore, AR target genes of crucial relevance to spermatogenesis should be sought in Sertoli cells (or other somatic cells in the testis). However, such genes have not been identified in vertebrates so far.

Androgens like T can be converted (aromatized) to estrogens and estrogen receptor (ER) expression has been detected in the vertebrate testis (reviewed by O'Donnell *et al.*, 2001; Socorro *et al.*, 2000; Wu *et al.*, 2001). However, ER-dependent signalling may not be of primary relevance to spermatogenesis in mice, since mutational ablation of the enzyme required for oestrogen synthesis resulted in an only partial impairment of spermatogenesis that started to develop well after puberty (Robertson *et al.*, 1999). Nonetheless, *in vivo* and *in vitro* studies indicated that estrogens can stimulate the proliferation of undifferentiated spermatogonia in eel (Miura *et al.*, 1999) and of gonocytes in rat (Li *et al.*, 1997), for example.

Genetic or experimental models with a specific failure of androgen-dependent signalling are not available yet in fish, but *in vivo* and *in vitro* studies have been carried out on the effects of androgens or LH-like hormones on spermatogenesis in fish. Treatment with gonadotropin preparations induced precocious spermatogenesis in a number of species (*e.g.* Funk & Donaldson, 1972; Khan *et al.*, 1987; Budworth *et al.*, 1994). Also androgen treatment induced spermatogenesis in a number of teleost species (*e.g.* Kobayashi *et al.*, 1991). Experiments in juvenile African catfish showed that treatment with 11-KT induced precocious spermatogonial proliferation and meiosis as well as development of secondary sexual characteristics (Cavaco *et al.*, 1998). Using a long-term testis tissue culture system, it could be shown that 11-KT promoted full spermatogenesis in the Japanese eel (Miura *et al.*, 1991^b). It appears therefore that in fish, spermatogonial proliferation is a prominent target of androgen action, in particular of 11-KT. The strong stimulatory effect of this androgen on testis growth might also reflect an action on Sertoli cell proliferation; interestingly, androgen-induced increases in Sertoli cell numbers have recently been described in the mouse (Haywood *et al.*, 2003).

Testosterone, on the other hand, inhibited the natural onset of spermatogenesis in African catfish and co-treatment experiments revealed that T was able to block 11-KT-induced spermatogenesis as well as the development of the seminal vesicles (Cavaco *et al.*, 2001). It appears that the steroidogenic output of the

testis requires being balanced precisely between T and 11-KT in order to prevent adverse effects of T on 11-KT-stimulated spermatogenesis. Since T treatment suppressed FSH plasma levels in salmon (Dickey & Swanson, 1998), it is possible that the inhibitory effect of T on spermatogenesis is based on interfering with FSH-dependent signalling.

Concluding Remarks

Androgens and FSH are the most important reproductive hormones as regards the regulation of spermatogenesis. The hormones' foremost function is to enable the Sertoli cells creating a microenvironment that responds to the germ cells' changing needs during their development. In some salmonid species, quantification of circulating FSH and LH (Swanson *et al.*, 1989; Prat *et al.*, 1996; Gomez *et al.*, 1999) showed that FSH plasma levels are clearly detectable, and seem transiently elevated, during the initiation of spermatogenesis; maximum levels are observed during rapid testicular growth, which then decline towards spawning. Plasma LH is undetectable or very low during spermatogonial proliferation and the initiation of meiosis, starts to increase during rapid testicular growth, and peaks at spawning. Considering that FSH stimulated steroidogenesis in immature and maturing testis (Planas & Swanson, 1995), and that only Sertoli cells carried a gonadotropin receptor that bound FSH (but also LH; Yan *et al.*, 1992; Miwa *et al.*, 1994), it appears possible that FSH signalling might be sufficient to initiate spermatogenesis by activating both Leydig and Sertoli cell functions. Two androgens seem to be involved in these processes: T exerts feedback effects (*e.g.* inhibiting FSH release or steroidogenesis), and might – after conversion to E2 – play a more direct role in the testis by stimulating stem cell renewal (Miura *et al.*, 1999). 11-KT stimulates Sertoli cell functions that in turn promote spermatogonial proliferation and further steps along the process of spermatogenesis (Miura *et al.*, 1991^b). Some of the effects on early stages of spermatogenesis involve 11-KT-stimulated growth factor production by Sertoli cells. However, the signalling molecules involved in mediating the effects of 11-KT in meiotic and postmeiotic germ cells require further research. Concerning the regulation of 11-KT production during these later stages, the LH plasma levels that start to increase during the rapid testicular growth phase, are likely to become more important for supporting the progressively increasing 11-KT production (Schulz & Blüm, 1990; Planas & Swanson, 1995).

It is also important to realize that a differential responsiveness exists towards FSH and androgens among different spermatocysts. For example, the presence of two adjacent spermatocysts containing germ cells in widely differing stages of spermatogenesis, although having been exposed to a similar endocrine environment, indicates that the response to a given endocrine signal varies from cyst to cyst. This local variation may involve a locally changing expression of growth factors, and growth factor and hormone receptors.

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