Puberty in Male Atlantic Cod (*Gadus morhua*)

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Fernanda Ferreira Loureiro de Almeida

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ISBN: 978-90-39350713

Cover illustration: Yuri J. van Overmeeren; photo: Norwegian Seafood Export Council

Printed by: PrintPartners Ipskamp, Enschede

The study described in this thesis was developed at the University of Utrecht, Faculty of Sciences, Department of Biology, division of Endocrinology and Metabolism and at the Institute of Marine Research, Austevoll Reasearch Station, Norway. This study was supported by the Norwegian Research council (grant 159662/S40).

Puberty in Male Atlantic Cod (*Gadus morhua*)

Puberteit van de mannelijke kabeljauw (*Gadus morhua*)

(met een samenvatting in het nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof. dr. J. C. Stoof, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op maandag 15 juni 2009 des ochtends te 10.30 uur

door

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geboren op 14 oktober 1976

te Corumbá, Mato Grosso do Sul, Brazilië

Promotor: Prof. dr. D.G. de Rooij

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À minha família

Irajá, Judith, Octávio (mano) e Janina (pipoca)

“As we Londoners ate our fish-and-chips from its newspaper wrappings, which of us would have believed that cod had started wars, founded cities, and kept the whole population of some northern countries alive? Who would have thought, as we fed cheap codfish heads to our cats, that cods’ cheeks, tongues and lips were delicacies in far off Iceland? And who now, seeing the rows of bottles of cod-liver-oil capsules in the local health food shop, would believe that cod, once the most abundant, prolific and hardy of fish, is in danger of disappearing from our seas?”

Mark Kurlansky in *Cod: A Biography of the Fish that Changed the World*

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1

Introduction

1. Introduction

1.1 Atlantic Cod: General Biology and Habitat

Teleost fish represent the most numerous group of vertebrates, comprising over 29,000 described species. They are distributed in the waters all over the globe showing a marvellous variety of morphological, physiological and behavioural adaptations (Turner 1993; Goodwin *et al*. 2002). Among them, the Atlantic cod (*Gadus morhua*, L) is a fish of the family Gadidae, within the order Gadiformes (Table 1), which includes species as haddock, whiting, pollock and coalfish (Scott and Scott 1988; Cohen *et al*. 1990; Kurlansky 1999). Atlantic cod can grow to 2 m (commonly from 30 to 100 cm) weighing up to 95 kg, with elongated body and moderately deep caudal peduncle (Figure 1), coloured normally in muted gray, green or brown with spots, an off-white belly (Stoskopf, 1993) and can live more than 20 years (Bigelow and Schroeder 1953; Scott and Scott 1988).

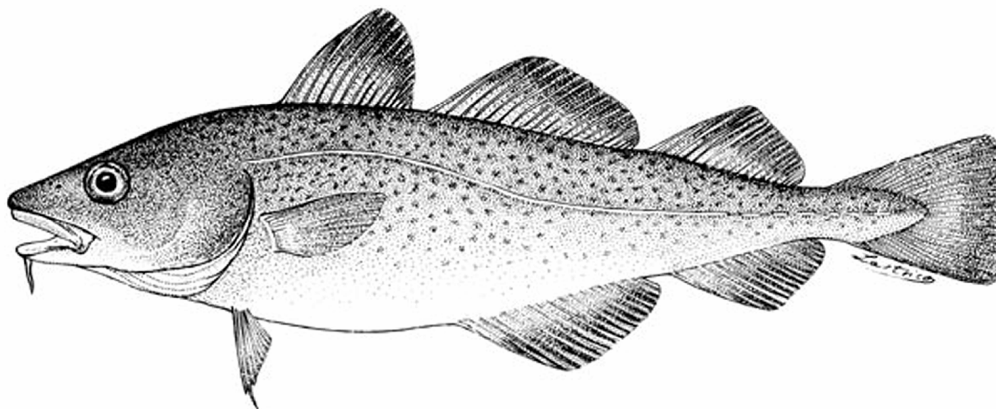

Figure 1 The Atlantic cod *Gadus morhua*, FAO Species/Identification Guide

Primarily pelagic (do not live close to the sea bottom), cod become demersal (bottom-dwelling) when feeding and spawning, being found mostly over continental shelfs at 150 to 200 m depth, although they have been trawled in depths up to 500 m (Lear 1993; Brander 1994a; Morgan *et al*. 1997; Smedbol and Wroblewski 1997). Cod are gregarious during the day, forming compact schools that swim between 30 and 80 m above the bottom, and scatter at night. Adapting to the most readily available food source, cod consume anything in their path. The fish have a barbell protruding from their lower jaws, which is used for finding prey buried in the sand. Atlantic cod is able to withstand prolonged periods of fasting, being able to survive up to 16 weeks deprived of food, specially during winter months, when there is a natural

decrease in food availability (Beaulieu and Guderley 1998). During prolonged fasting, they slow down their metabolism and use mainly lipids and glycogen stored in the liver as energy source (Hemre *et al*. 1993a, b).

Table 1 Taxonomic hierarchy and nomenclature of Atlantic cod.

Scientific name	*Gadus morhua* Linnaeus, 1758 (TSN: 164712)
Kingdom	*Animalia*
Phylum	*Chordata*
Subphylum	*Vertebrata*
Superclass	*Osteichthyes*
Class	*Actinopterygii*
Subclass	*Neopterygii*
Infraclass	*Teleostei*
Superorder	*Paracanthopterygii*
Order	*Gadiformes*
Family	*Gadidae*
Subfamily	*Gadinae*
Genus	*Gadus*
Species	*Gadus morhua*
Vernacular names:	Atlantic cod (English) Morue de l'Atlantique (French) Bacalao del Atlántico (Spanish) Bacalhau (Portuguese) Kabeljauw (Dutch) Treska (Russian) Torsk (Danish; Norwegian) Dorsch, Kabeljau (German)

Font: Integrated Taxonomic Information System – ITIS North America

The natural habitats of this omnivorous species are the Northern Atlantic coastal regions. Populations of Atlantic cod are found across the Northern Atlantic Ocean, from the gulf of Maine, USA, in the West, North past Greenland and Iceland to the Baltic Sea and South to the Bay of Biscay (Cohen *et al*. 1990) with, however, little or no interbreeding between the different populations. Stocks of cod generally move from coastal to offshore areas in autumn, over winter in deep slope waters, and return to more inshore areas in the spring (Woodhead 1975). In the Northern Atlantic Ocean, they can be found over a wide range of salinities from almost fresh to full oceanic water. Water temperatures range from -2 to 20^{0}C, but the presence of cod usually depends on prey distribution rather than on temperature (Cohen *et al*. 1990; Sundby 2000).

Seasonal migrations of cod are photoperiod-triggered and modulated by hormones, in particular thyroid hormones, which enhance metabolism, sensory physiology and swimming capacity during migrations (Comeau *et al*. 2001). These migrations, which can cover distances of up to 1000 km and average 5 km per day, are well-known for different cod populations, from Arcto-Norwegian and Barents Sea cod (Harden Jones 1968, Woodhead 1975; Ottera *et al*. 1999) to Gulf of St. Lawrence cod, and Southern Gulf of St. Lawrence cod (Jean 1964; Paloheimo and Kohler 1968; Templeman 1979; Moguedet 1994; Taggart *et al*. 1995).

1.2 Cod in Human History

Cod has long been part of human history. English, French, Spanish and Portuguese fleets sailed to Newfoundland for cod already more than four centuries ago. The fish were split, salted and dried, or pickled in brine to be preserved. So many were consumed that North American cod is a staple of traditional French, Portuguese, Spanish and Italian cuisine to this day, as the colonist in North America not only ate cod themselves, but also exported it to Catholic Europe and to Caribbean island plantations (Kurlansky 1999).

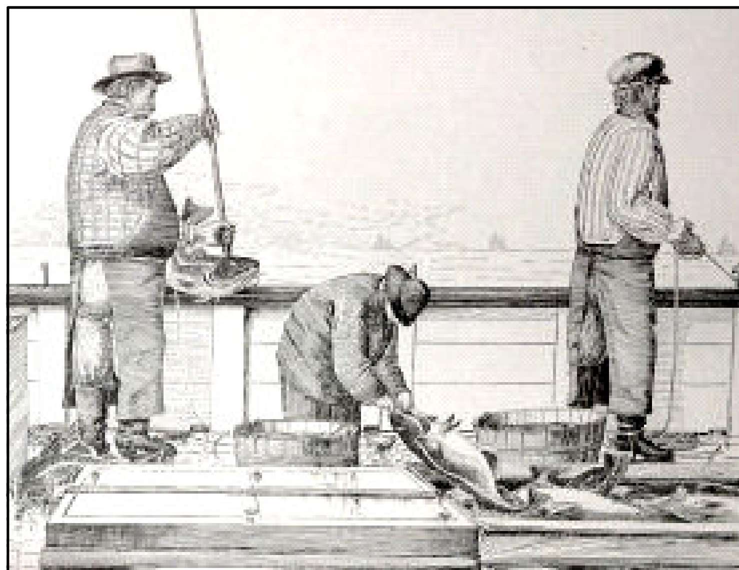

Figure 2 Gaffing and cleaning cod on the deck of a hand lining schooner off the North American east coast, ca. mid nineteenth century.

Ground fishing was the first colonial industry in America (Figure 2) and the nineteenth-century cod fishing was a community activity that involved all members from the family (Vickers 1994). With the population growth and expansion towards the American West, salted cod went west carried on wagon trains, as at that time it was cheaper to relay in salty fish than

locally grow beef or pork. Moreover, in American South, the majority of plantation managers bought dried cod to feed their slaves. Cod founded thriving cod-aristocracies, particularly in cities like Boston and Massachusetts (Kurlansky 1999). The Industrial Revolution caught up with the fishing industry at the century turn, with the introduction of steam powdered vessels historically changing how bottom dwelling fish were caught.

By extracting catch and fishing data from historical logbooks, an estimation of the cod population on the Scotian Shelf off the North America East coast in 1852 was calculated and compared to population estimates of cod today. The study demonstrates a twenty fold decrease of cod abundance on the Scotian Shelf from 1852 to the present (Rosenberg *et al*. 2005). Moreover, several stocks of cod in the Northwest Atlantic collapsed in the early 1990s (decline by more than 95% of maximum historical biomass) and have failed to respond to complete cessation of fishing (in 2005 the biomass of these stocks increased only slightly, ranging from 0.4 to 7.0%) (Frank *et al*. 2005). So far, governments across the North Atlantic are struggling to rebuild depleted cod stocks (Rosenberg *et al*. 2005) and very recently a slow increase in stock of Northeast Arctic cod was reported (Bogstad 2009).

1.3 Industrial Cod Farming

As shown above, Atlantic cod represents, historically, an economically important fish; his flesh with a mild flavor, low fat content and a dense white protein is considered the "beef of the sea" and can be marketed fresh, chilled, frozen, salted, smoked and in brine (Innis 1978). Moreover, other commercially valued products are also obtained from cod, such as salted cheeks, liver oil and eggs. Consequently, there has been an over fishing of the cod wild populations, especially during the last century, and the species is classified as vulnerable to extinction since 1996 by the World Conservation Union (Solbel 1996) and Outside Safe Biological Limits by the International Commission for the Exploration of the Seas (ICES 2003).

With the gap widening between increasing demand and diminishing cod fishery supply, the interest in farming this species has been growing especially in Norway, Iceland, Scotland, Faroe Islands and Canada (Brown *et al*. 2003; Moksness *et al*. 2004) and Atlantic cod is currently the primary species being developed for commercial culture.

With some exceptions, culture of gadoids is found in the same areas were also the salmon farming industry is established in Norway, the United Kingdom, and on the East coast of the USA and Canada, and the West coast of Chili (Table 2). In addition, smaller operations are active in Iceland and Spain (Rosenlund and Skretting 2006). Norway has gone farthest in developing cod farming industry, registering in only one year (2005-2006) an increase of 1.8 fold in ton of produced cod (Karlsen *et al*. 2005). With 16 commercial hatcheries operating in 2006

and more than 500 cod farming licenses issued (Norwegian Seafood Centre, Bergen, Norway) totalizing a production volume of 4 x 10^6 m^3, the estimation for the production capacity of cod in Norway is about of 300,000 ton/year (Rosenlund and Skretting 2006).

Table 2 Overview of production of gadoids in 2003.

Country	Species	Number of Hatcheries	Juveniles (10^3)	Harvested Fish (t)
Canada & USA	Atlantic cod (*Gadus morhua*)	3	500	< 1,000
Chili	Hake (*Merluccius australis*)	1	400	Not known
Spain	Pollock (*Pollachius pollachius*)	1	200	< 200
Iceland	Atlantic cod	1	250	Not known
United Kingdom	Atlantic cod	2	400	< 100
Norway	Atlantic cod	16	4,000	1,500

Font: Rosenlund and Skretting 2006

This development is possible because the potential of cod farming is very high due to the species ' biological features, such as short egg and larval stages (cod larvae have a very high growth potential, reaching 1 g in 70-80 days after hatch; Otterlei *et al*. 1999), rapid growth, good feed utilization and satisfactory behavior. Moreover, cod adapt very well to conventional sea cages. Most of the challenges offered by cod farming generally concern nutrition, such as larval feeding, cannibalism in young phase, lipid deposition in the liver and also premature sexual maturation (Knutsen and Tilseth 1985; Kjørsvik *et al*. 1991; Folkvord 1991).

Juveniles are being produced year-round in hatcheries, selective breeding is underway and vaccines are being developed (Björnsson, in press). Initially, the juveniles are reared in land-based farms where optimal temperatures can be maintained, but for the rest of the life-cycle cod grow in sea pens at ambient temperature. Temperature is one of the most important environmental cues determining the potential growth of cod (Brander 1994b; Björnsson *et al.* 2001). Consequently, the feasible locations for cod farms depend largely on sea surface temperatures. In the already named countries where cod farming is being developed, the lowest and the highest monthly mean water temperatures range between -1 to 14^0C (Jónsson 2004).

Although some knowledge is already available and cod industrial production keeps increasing, cod farming still presents significant challenges. Farmed cod (Norwegian Coastal cod) when escaping from cages, which occurs more often with cod than with Atlantic salmon (Moe *et al*. 2007), can survive and, in addition, interact with the natural ecosystem (Kristiansen and Svåsand 1990). For example, interbreeding between wild and escaped farmed fish can

result in genetic changes in the wild populations that reduce overall fitness and productivity (Utter *et al*. 1993; Utter 1998). In addition, escaped farmed cod may transmit pathogens to wild populations (Heuch *et al*. 2005; Øines *et al*. 2006). Moreover, as an exclusively marine species, cod can spawn in the net pens when reaching sexual maturation before harvesting. Using genetic markers, Jørstad *et al* (2008) showed that 20% of cod larval population in open waters in the vicinity of cod farms originated from farmed fish that had spawned in the sea net cages. Escapes of fish from aquaculture installations as well as spawning in net sea cages are regarded as major risk factors for negative genetic impacts on native gene pools.

1.4 Cod Reproduction

As in many other fish living at high latitudes, the life-cycle of Atlantic cod follows a seasonal pattern. Although the exact time of the spawning period varies among the populations, gonadal development starts in late summer in most cod in the Eastern and Western parts of the North Atlantic, lasting approximately 6 months before the annual spawning commences in early spring (Burton *et al*. 1997). After spawning, in late spring/early summer, the recovering adults migrate to rich summer feeding grounds to regain and store energy. The re-start of gametogenesis, which initiates the following reproductive cycle, also signals the start of the migration back towards the spawning grounds during winter (Cohen *et al*. 1990; Rideout and Burton 2000; Rideout *et al*. 2000). Among the various environmental cues changing over time, such as temperature, food supply or photoperiod, the latter is well known for modulating sexual maturation in cod. Experimental alteration in daylenght delays gonadal development and gametogenesis for months in animals kept in sea cages, arrests gonad development in indoor tanks, or pushes forward spawning when photoperiod changes are compressed (Karlsen *et al*. 2006; Skaeraasen *et al*. 2004; Taranger *et al*. 2006; Hansen *et al*. 2001; Norberg *et al*. 2004; Davie *et al*. 2007; Dahle *et al*. 2000).

Egg production in cod, as in all egg-laying vertebrates, is a time-consuming process where energy is transferred from the liver to the developing oocytes. Cod is a multiple batch spawner and there is no recruitment of oocytes to the vitellogenic stage during the spawning season (Kjesbu *et al*. 1990; Kjesbu 1993). Thus, the number of oocytes produced in a spawning season is determined prior to the onset of spawning, so that it is possible that energy reserves available at a particular time strongly determine the final egg production. Indeed, egg production is related to body weight and length in the 3 - 4 months before the onset of spawning, probably during the onset of vitellogenesis (Skæraasen *et al*. 2005). Moreover, even before the initiation of vitellogenesis, females showing greater growth have higher oocyte numbers (Kjesbu *et al*. 1991). Individual females spawn over a period of approximately 50 to 60

days, releasing from 9 to 20 egg batches of about 300,000 eggs at intervals of 60 to 70 hours (Kjesbu 1989; Trippel 1998). Cod are described as one of the world's most fecund fish, releasing, in general, from 250 to 500 thousands eggs/kg over a typical spawning season (Cohen *et al*. 1990; Walden 2001).

The act of spawning happens preferentially in the evening or at night (Ferraro 1980; Kjesbu 1989) and is preceded by a mating ritual in which females select courtship displaying males. Female mate choice is based on a combination of male grunting, a drumming noise generated by a muscle associated with the swimming bladder, and fin display, reflecting the male "status" and quality (Brawn 1961; Engen and Folstad 1999; Hutchings *et al*. 1999). After selection, the pair initiates spawning by entering a ventral mount that permits a close alignment of the urogenital openings and consequently achieving high fertilization rates (Brawn 1961; Engen and Folstad 1999). Unfertilized eggs and sperm remain viable for more than one hour in seawater (Kjørskvik and Lønning 1983; Trippel and Morgan 1994), though the sperm motility decreases with time (Trippel and Neilsen 1992; Litvak and Trippel 1998).

The time for the eggs to hatch varies with temperature; it normally occurs 80 to 100 degree days (measure of temperature variation within time variation) post fertilization (Laurence and Rogers 1976; Brown *et al*. 2003). Cod larvae are only a few millimeters long (from 3.5 to 4.5 mm; Walden 2001) and motionless when they hatch, drifting approximately in the top 50 m of the water column (Hall *et al*. 2004). Initially, they do not feed, relaying on the yolk sac for nutrition, but due to their limited yolk reserve, they must begin to eat 3 to 6 days after hatching (Walden 2001; Hall *et al*. 2004). Larval and juvenile growth is temperature dependent (Otterlei *et al*. 1999). Juvenile cod spend their first year of life on nursery grounds, after which they join the adult populations in the complete maturation cycle from 2 to 3 years post-hatch until puberty (Cohen *et al*. 1990).

In different Atlantic cod populations, drastic declines in both age and length-at-maturity were observed in the last decade, not only as a compensatory response to lower population density, but also as a size-selectivity for early maturation (genetic selection) provoked by fishing pressure (Trippel 1995; Morgan and Brattey 1996; Saborido-Rey and Junquera 1999; Nash *et al*. 2005). Decrease in age and length at maturation represents a negative impact for the fecundity of Atlantic cod, since egg diameter is positively correlated with length of the female (Kjesbu 1989) and duration of spawning and fecundity increase with age (Buzeta and Waiwood 1982; Hutchings and Myers 1993).

Female gamete development demands higher quantities of liver-stored lipids than male to enter rapid spermatogonial proliferation, as lipids are directly used in the synthesis of vitellogenin (Tyler and Sumpter 1996). This high critical threshold of energy may be the reason

why females attain puberty later than males (Taggart *et al*. 1994; Morgan and Brattey 1997). This situation makes the problems related to precocious puberty more relevant in males than in females. In addition, females present higher growth rate than males, reaching market size before their counterparts. Therefore, efforts to prevent Atlantic cod gonad maturation are mainly directed towards males.

1.5. Spermatogenesis in Teleosts

Spermatogenesis is a cyclic, complex and highly coordinated process that encompasses the formation of spermatozoa from undifferentiated spermatogonia. The process is fueled by spermatogonial stem cells that provide the basis for male fertility. During spermatogenesis, germ cells undergo proliferation and differentiation steps. Spermatogenesis starts with a mitotic proliferation phase, resulting in a greatly expanded spermatogonial population when approaching meiosis with the formation of preleptotene primary spermatocytes. This is followed by the long prophase of the first meiotic division, during which homologous chromosomes pair and the primary spermatocytes increase in size and display distinct nuclear morphologies as they pass through leptotene, zygotene, pachytene and diplotene. The first meiotic division is completed after a rapid metaphase, anaphase and telophase, giving rise to secondary spermatocytes that proceed immediately and rapidly through the second meiotic division to form haploid spermatids. Finally, spermatids undergo a cellular transformation (spermiogenesis) until they are ultimately released as functional flagellated spermatozoa (Russel *et al*. 1990; Schulz *et al*. 2009). This cellular development is tightly regulated and requires a special microenvironment created by a somatic cell type, the Sertoli cell, which forms cytoplasmic extensions enveloping the germ cells. Germ cells and Sertoli cells are present in the seminiferous tubules and form the germinal epithelium of the testis parenchyma. Germ cell survival, proliferation and differentiation critically depend on this continuous interaction with the Sertoli cells that provide structural and physiological support, involving paracrine and hormonal interactions between these two cell types. It is now clear that the specialized functions required for proper proliferation and differentiation of the spermatogonial stem cells are mainly provided by the neighboring Sertoli cells, and the "niche" created by them controls the balance between renewal and differentiation of the stem cells (Spradling *et al*. 2001). Besides the germinal compartment, testis parenchyma also contains the interstitial tissue, with the androgen-producing Leydig cells, blood vessels, macrophages, connective and nervous tissue elements (Russell *et al*. 1990).

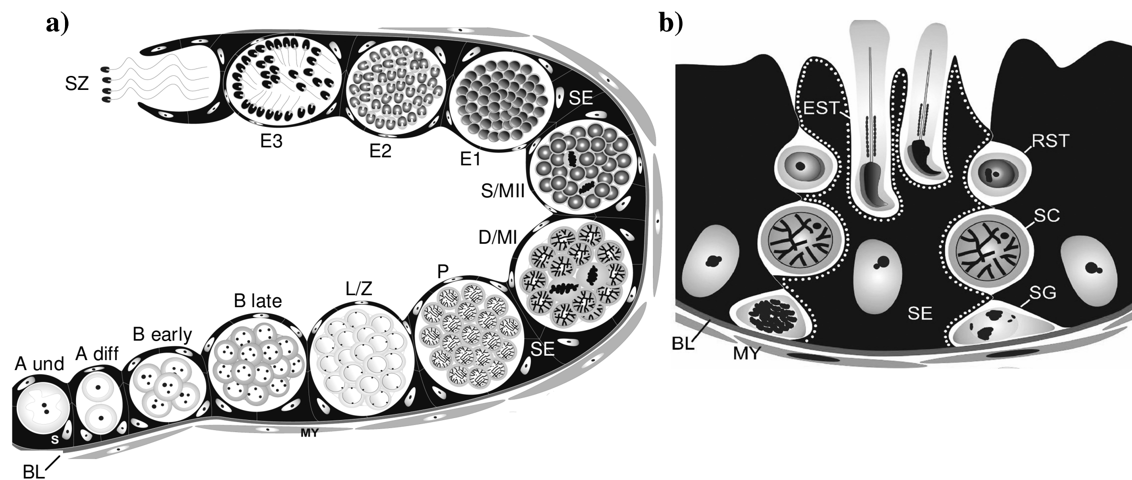

Figure 3 Schematic representation of the germinal epithelium of cystic (**a**; found in fish and amphibia) and non-cystic (**b**; found in reptiles, birds and mammals) spermatogenesis containing Sertoli (**SE**) and germ cells, delineated by a basal lamina (**BL**) and peritubular myoid cells (**MY**). In a) Type A undifferentiated spermatogonia ($\mathbf{A_{und}}$); type A differentiated spermatogonia ($\mathbf{A_{diff}}$); 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**). In b) Spermatogonia (**SG**); spermatocyte (**SC**); round spermatid (**RST**); and elongated spermatid (**EST**). Adapted from Schulz *et al.* 2009.

Although the general characteristics of spermatogenesis are similar in all vertebrates, the relation between germ and Sertoli cells differs in fish and amphibian on the one hand, and reptiles, birds and mammals on the other hand. Spermatogenesis in teleosts and amphibians develops in spermatogenic cysts formed by cytoplasmic extensions of Sertoli cells, within the spermatogenic tubules, containing the descendants of a single spermatogonial stem cell. Consequently, the germ cells of a given cyst proceed through spermatogenesis synchronously within the cyst (Figure 3a). Different from mammals, birds and reptiles, in cystic spermatogenesis not all germ cells from a cyst are surrounded by cytoplasmic extensions of the Sertoli cells, which delineate the cysts. Each cyst starts with an association between one or two Sertoli cells and an undifferentiated type A spermatogonium (Pudney 1993; Schulz *et al*. 2005). When differentiating, the type A spermatogonium enters mitosis to form a pair of spermatogonia that divide synchronously thereafter to constitute an isogenic germ cell clone bordered by the cytoplasmic extensions of a single layer of Sertoli cells, forming the spermatogenic cyst. Thus, in cystic spermatogenesis, a given Sertoli cell, which proliferates also after puberty (Miura 1999; Schulz *et al*. 2005; Leal *et al*. 2009), is usually in contact with only a single germ cell clone that is accompanied through the different stages of spermatogenesis by its associated group of Sertoli cells (Grier 1981; Hess *et al*. 2005). This is in contrast to spermatogenesis in birds, reptiles and mammals, where at any given time a Sertoli cell contacts members of different germ cell clones that are in different stages of spermatogenesis (Figure

3b). Spermiation, the release of mature germ cells by Sertoli cells, is achieved by the cysts opening. The cystic type of spermatogenesis, in which Sertoli cells nurse only one germ cell type/clone at a time, makes fish an interesting model for studying the regulation of spermatogenesis. For instance, recent studies have used the cystic model of fish spermatogenesis for laser-capture microdissection of germ cells from the same cyst (same stage), enabeling the authors to analyse gene expressions in germ cell types in different developmental stage (Viñas and Piferrer 2008).

A crucial phase during spermatogenesis is the period of spermatogonial proliferation, an important target of different regulatory processes of spermatogenesis, while meiosis and spermiogenesis are strictly conserved, relatively inflexible processes not allowing much regulation, except perhaps for the incidence of apoptosis. Precise knowledge about the number of mitotic divisions that spermatogonia undergo is essential for understanding the regulatory mechanisms targeting spermatogenesis (de Rooij and Russell 2000; Ando *et al*. 2000). Spermatogonial cells give rise to spermatocytes after a fixed number of cell divisions characteristic of each species. Hence, the number of mitotic divisions of spermatogonia preceding meiosis is species-specific and therefore genetically determined (de Rooij and van Dissel-Emiliani 1997; de Rooij 2001; Ando *et al*. 2000). Studies show that the number of spermatogonial generations ranges from six to 16 in teleost species (Table 3).

Table 3 Number of spermatogonial generations in teleost species.

Species	Name	Number of Spermatogonial Generations	Reference
Hucho perryi	Japanese Huchen	6	Ando *et al*. 2000
Salvelinus leucomaenis	White-spotted Char	6	Ando *et al*. 2000
Oncorhynchus masou	Masu Salmon	8	Ando *et al*. 2000
Carassius auratus	Goldfish	8	Ando *et al*. 2000
Oreochromis niloticus	Tilapia	8	Vilela *et al*. 2003; Schulz *et al*. 2005
Cheilodipterus lineatus	Cardinal fish	8	Fishelson *et al*. 2006
Apogon hungi	Cardinal fish	8	Fishelson *et al*. 2006
Danio rerio	Zebrafish	9	Leal *et al*. 2009
Astatotilapia flaviijosephi		8-10	Fishelson 2003
Oryzias latipes	Medaka	8-10	Shibata & Hamaguchi 1988; Ando *et al*. 2000
Anguilla japonica	Japanese Eel	10	Miura *et al*. 1991
Poecilia reticulata	Guppy	14	Billard 1969
Tilapia zillii	Red belly Tilapia	16	Fishelson 2003

Another important parameter used to evaluate spermatogenesis is the gonadosomatic index (GSI = gonad weight * 100/ body weight), since testicular weight gain in seasonally reproducing species is mainly attributable to germ cell production during spermatogenesis. Furthermore, the gonadal weight increase demands energy, leading to a decrease in food to flesh conversion and body growth, making the GSI an important parameter to be evaluated not only in experimental conditions but also in the aquaculture industry.

During the spermatogenic process, germ cell loss via apoptosis occurs in all species investigated and plays a critical role in determining the amount of sperm output in mammals (Roosen-Runge 1973; Sharp 1994; Baum *et al*. 2005). As much as 75% of the spermatozoa that theoretically could be produced are eliminated through apoptosis in mammals (França and Russell 1998; de Rooij and Russell 2000). Germ cell death occurs, exclusively or preferentially, in certain developmental stages, with variations between species in quality and quantity (Roosen-Runge 1977). For example, in many rodents, apoptosis occurs mainly during the spermatogonial phase, referred to as density-dependent regulation, but it can also occur during meiosis, probably related to chromosomal damage, functioning as quality control system during monitoring chromosomal integrity (Braun 1998). In seasonally breeding newt the degeneration of germ cells is observed at the transition from spermatogonia to spermatocytes (Yazawa *et al*. 2000). In fish, the situation seems more diverse than in tetrapods; in tilapia, for example, germ cell apoptosis incidence is high during spermiogenesis (Vilela *et al*. 2003; Schulz *et al*. 2005); in guppy and zebrafish, apoptosis is observed mainly during the mitotic phase of – in part – late spermatogonia (Billard 1969; Leal *et al*. 2009); in the cartilaginous fish *Torpedo marmorata*, apoptosis affects in particular spermatocytes and spermatids (Prisco *et al*. 2003) and in the dogfish (*Squalus acanhtias*) germ cell degeneration occurs especially among late spermatogonia and early spermatocytes (Dodd *et al*. 1960; Callard *et al*. 1995; Callard *et al*. 1998). Despite the intercellular bridges between cells belonging to the same clone, not all members of the same clone enter in apoptosis (Hamer *et al*. 2003), indicating that it is rather a developmental problem of an individual germ cell than a problem of the cyst-forming Sertoli cells. Moreover, in seasonally breeding fish, germ cells enter a degenerative process after the breeding season and the tubules are reorganized during removal of residual spermatozoa, in preparation for the subsequent wave of development (Besseau and Faliex 1994; Chaves-Pozo *et al*. 2005). Sertoli cells are involved in germ cell elimination in teleosts both during spermatogenesis and in the post-spawning "clean-up" of the tubules (Billard and Takashima 1983; Scott and Sumpter 1989). During apoptosis, the cells shrink and exhibit several typical features such as cell membrane disruption, cytoesqueletal rearrangement, nuclear condensation and internucleosomal DNA fragmentation. The presence of internucleosomal chromatin

degradation in the majority of apoptotic cells is one of the best characterized biochemical features of apoptotic cell death and has resulted in the use of DNA fragmentation as a diagnostic tool for the occurrence of apoptosis (Kaufmann and Hengartner 2001; Nagata 2000). For instance, the TdT-mediated dUTP nick-end labelling (TUNEL) is being used routinely to detect apoptotic cell in various tissues (Sinha-Hikim and Swerdloff 1999).

1.6 Onset of Male Puberty in Teleost

Teleost fish represent the largest group of vertebrates; therefore it may not be surprising that they display a broad variety of reproductive strategies involving different features of energy requirement, for example as reflected in the presence, absence or different degrees of reproductive-associated migratory behaviour or in large differences in gonad weight between species. Similarly, mating and paternal behaviour, gamete structures and specificity in recognition of surface molecules of eggs and sperm vary greatly among the nearby 30,000 species (Breder and Rosen 1966; Baylis 1978; Hart 1990; Turner 1993). Despite this, the biological processes constituting gonadal maturation and the regulatory mechanisms involved are of great similarity, or show small variation within the same main scheme, in all studied teleost. Therefore, revealing specific species features contributes to the knowledge about the reproductive biology of the species on one hand (and may be of applied value as well), but at the same time can contribute to understanding basic principles underlying the regulation of reproduction.

In many teleost species that live at high latitudes, exogenous environmental signals, like temperature, photoperiod or food availability, trigger activity changes in the brain-pituitary axis that affects the timing of pubertal gonad maturation. The external signals are suspected entraining neuroendocrine signalling to the hypothalamus that, in turn, secretes gonadotropin releasing hormone, which reaches the pituitary gland in fish by a direct innervation from the hypothalamus (Redding and Patino 1993). The stimulated pituitary secretes the two gonadotropins, follicle stimulating hormone (Fsh) and luteinising hormone (Lh) that will stimulate specific receptors expressed by the somatic cells in the testis. Consequently, remarkable anatomical, cytological and functional changes occur in the testis in context with pubertal activation of the two main functions of the testis, namely spermatogenesis and sex hormonal and growth factor production.

This brain-pituitary axis has evolved in vertebrates as the hormonal master control system over spermatogenesis, and it provides adaptive options. For example, it suppresses puberty until somatic development has proceeded sufficiently to cope with the pressure related to reproduction. Moreover, in adults, reproductive activity can be restricted to certain periods

(reproductive seasons) or can be suppressed altogether, under unfavorable conditions. This exemplifies the orchestration of the activities of the reproductive system with environmental conditions on the one hand, and with the developmental and physiological state of the individual on the other hand (Schulz and Miura 2002).

1.7 Aim and Outline of This Thesis

With the increasing dilemma of finding an acceptable and sustainable equilibrium between human exploitation of natural resources (such as over fishing) on the one hand, and biodiversity and conservation biology (cod strains and cod as an important species in the marine ecosystem) on the other hand, a potential solution is the cod farming industry.

However, as often encountered in industrial farming of any species, cod aquaculture presents its challenges and therefore optimization of the system, especially of the growing phase, is warranted. Some of these problems are the exploitation of other species that are turned into cod food and the genetic impact that escaped fish and spawning in sea cages can cause to local wild cod populations. For example, early puberty attenuates sustainability through losses in flesh quality and growth performance, and also increases the risk of unwanted reproduction in aquaculture facilities.

These considerations are societal and biotechnological reasons contributing to the relevance of the present work. The main focus of the present project was to study the pubertal maturation in male Atlantic cod and its regulation by the photoperiod, at an intersection between basic and applied research. In this context, the understanding of photoperiod-modulation of cod testicular maturation is desired for fundamental and comparative interests, and also it is of significance for the aquaculture industry of cod, in which an ability to modify the timing of puberty would be of great economic value.

To achieve this goal, at first a detailed histological study of cod testicular parenchyma as well as of the spermatogenic process has been carried out and is presented in **Chapter 2**. Detailed information is lacking in the literature while a comprehensive knowledge of testis structure and spermatogenic process is of essential importance when analyzing the effects of experimental conditions, such as changes in the photoperiod, on male puberty. With this knowledge, it was possible to analyze the effects of experimental light conditions on cod spermatogenesis and androgen production (**Chapter 3**). In this study, approximately 840 males cod were reared in: a) simulated natural light during 17 months; b) constant artificial light during 17 months; c) simulated natural light for six months followed by artificial constant light for 11 months; and d) artificial constant light for six months followed by simulated natural light for 11 months. This large scale experiment resulted in grossly different testicular growth

patterns, spermatogenic development, as well as androgen plasma levels. As puberty is directly coordinated by the brain-pituitary axis and mediated via their hormonal control, **Chapter 4** presents the main effects of the above-described experimental light regimes on the expression of pituitary gonadotropins and their cognate receptors in the testis. Since androgens are known as crucial signaling molecules for spermatogenesis and male reproduction in general, the cod androgen receptor was cloned and characterized, and its expression was studied in testis tissue from two of the four above described photoperiod treatment regimes (**Chapter 5**). Finally, the summarizing discussion (**Chapter 6**) completes this thesis.

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2

Spermatogenesis in Atlantic Cod (*Gadus morhua*): A Novel Model of Cystic Germ Cell Development

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Biol Reprod, Jan 2008; 78: 27 - 34.

Spermatogenesis in Atlantic Cod (*Gadus morhua*): A Novel Model of Cystic Germ Cell Development

ABSTRACT

Precocious male puberty significantly compromises sustainability aspects of aquaculture in a number of finfish species. As part of a program aiming to understand and eventually control testis maturation in farmed Atlantic cod, we studied the first reproductive cycle. The gonadosomatic index shows a 41-fold increase from immature (August) to mature (March) stages, reaching almost 10% of the total body weight. The paired cod testes are composed of several lobes arranged around a central collecting duct. In each individual lobe, spermatogenesis occurs in a marked gradient of development, with undifferentiated spermatogonia in the periphery of the lobe and the most advanced germ cells in the vicinity of the collecting duct, suggesting a tight spatiotemporal organization of spermatogenesis in the testis lobes of this species. Spermatogonial proliferation starts in August and continues for about 6 mo. Meiosis and spermiogenesis are first observed in October and are completed in all cysts by February, when a 2-mo-long spawning season starts. Spermatogonia go through 11 mitotic divisions before differentiating to primary spermatocytes. Apoptosis is rare, but when observed it occurs mainly during the last spermatogonial generations. Our observations suggest a model in which a maturational wave progresses through each growing lobe that is first driven by appositional growth from the lobe's periphery, reflecting spermatogonial proliferation and cyst formation which, when ceasing, is terminated by completing spermiogenesis and spermiation that progress toward the lobe's periphery.

INTRODUCTION

Spermatogonial stem cells can either self-renew or produce spermatogonia committed to the developmental steps constituting spermatogenesis [1]. Via mitotic proliferation of spermatogonia, meiotic recombination in spermatocytes, and cellular differentiation of spermatids, mature haploid spermatozoa are formed [1, 2]. During spermatogenesis, the germ cells descending from a given stem cell stay interconnected via cytoplasmatic bridges, thereby constituting a synchronously developing germ cell clone. This sequence of developmental steps requires a specific microenvironment that is created by somatic cells, particularly the Sertoli cells. Germ cell survival and development critically depend on their continuous and close

contact to Sertoli cells that provide structural and physiologic support, including paracrine interaction between these two cell types. In anamniote vertebrates (fish and amphibians), cytoplasmic extensions of Sertoli cells envelop the individual germ cell clones, forming spermatogenic cysts, which together constitute the germinal epithelium in the seminiferous tubules of the testis [3–5]. Hence, the main difference to spermatogenesis in higher vertebrates is that in cystic spermatogenesis a given Sertoli cell usually is in contact only with a single germ cell clone.

A crucial phase during spermatogenesis is the period of spermatogonial proliferation. Precise knowledge about the number of mitotic divisions that spermatogonia undergo is essential for analyzing the regulatory mechanisms targeting spermatogenesis [6, 7]. Usually, a spermatogonium completes a species-specific, predictable number of mitotic cell cycles, before differentiating into spermatocytes, and hence entering meiosis; this number can vary between 2 and 14 in vertebrates [8].

During spermatogenesis, loss of a certain percentage of germ cells via apoptosis is normal in all species investigated, and it plays a critical role in determining spermatogenic efficiency [2, 9]. Germ cell death occurs exclusively or preferentially in certain developmental stages, also varying in a species-specific manner in quality and quantity [10]. For example, in rodents, apoptosis occurs mainly during the spermatogonial phase, referred to as density-dependent regulation, but can also occur during meiosis, related to irreparable chromosomal damage [6]. In teleosts, apoptotic germ cells have been observed mainly during the spermiogenic phase [11, 12]. Moreover, in seasonally breeding fish, germ cells not released during the reproductive season are phagocytized during the tubules' reorganization for the next season [13]. Sertoli cells are involved in both types of germ cell elimination in teleosts [14, 15].

The Atlantic cod (*Gadus morhua*, L.) is an economically important marine fish of the northern hemisphere. In recent years, natural stocks of Atlantic cod have been declining, and cod aquaculture has become increasingly relevant [16]. However, under farming conditions, male cod in particular commence and complete puberty much earlier than in the wild, and sexual maturation not only compromises flesh quality and growth performance [17] but also leads to unwanted introduction of genetic traits in wild populations via escaped mature fish or reproduction in aquaculture facilities, with the fry leaving the facilities. Attempts to prevent the initiation of testis maturation are an effective manner of controlling all aspects of unwanted reproduction, but they require understanding the regulation of this developmental process. Moreover, the cod belongs to an order of fish (gadiformes) in which spermatogenesis has not been studied in detail. We investigated (morphology, proliferation, apoptosis) the first

maturation of cod testis over a period of 1.5 yr and found a novel pattern of development of the spermatogenic process that is presented in this paper.

MATERIALS AND METHODS

Samples

A mixed population of male and female Atlantic cod of Norwegian Coastal Cod origin was reared in 7 m^3 seawater tanks under ambient light conditions at Austevoll Aquaculture Research Station, Norway (60^0N). The larvae were first fed on natural zooplankton in a semienclosed seawater pond at the Institute of Marine Research according to the method of Blom et al. [18] before transfer to the experimental tanks, where they were fed a commercial dry pellet diet *ad libitum*. All fish were treated and killed according to Norwegian National Legislation for laboratory animals. The water temperature ranged from 7.48^0C to 9.48^0C (mean ± SD = 8.1 ± 0.3^0C) during the experimental period.

Testis samples from 5–14 fish were analyzed (morphology, proliferation, apoptosis) monthly for 16 mo starting in July 2004, when the fish were 18 mo old (prepubertal). Body and testis weight was recorded to calculate gonadosomatic index (GSI = testis weight X 100 / body weight). Testis tissue was fixed in Bouin fluid by immersion, dehydrated, and embedded in paraffin according to conventional techniques. For all further studies, testis sections of 4 µm thickness were used.

The GSI values were $\log_{10}$-transformed and tested by one-way ANOVA, followed by Student-Newman-Keuls multiple comparisons test. A significance level of 0.05 was applied in the test.

Immunohistochemical Detection of Proliferation

Proliferation of spermatogonia and Sertoli cells was assessed by Phosphorylated histone H3 (pH3) immunodetection. Histone H3 is a chromosomal protein component involved in the condensation of mitotic and meiotic chromosomes and becomes phosphorylated during late G2 phase, being present until metaphase in the cell cycle [19, 20] (i.e., it is detectable in cells preparing to divide). For this purpose, the sections were mounted on glass slides coated with 3-aminopropyl triethoxysilane (TESPA; Sigma, St. Louis, MO), and dried overnight at 37^0C. The sections were deparaffinized and hydrated before incubation in a plastic chamber filled with 1 mM EDTA solution containing 0.05% Tween 20, pH 8.0 (Merck-Schuchardt, Hohenbrunn, Germany). For epitope retrieval, the glass chamber was transferred to a boiling water bath for

20 min and then left to cool to room temperature. Nonspecific protein binding sites were blocked with 5% goat serum (Vector Laboratories, Burlingame, CA) þ 1% BSA (Sigma) in PBS for 30 min, followed by an incubation with a polyclonal rabbit anti-human phosphohistone H3 IgG preparation (Upstate, Charlottesville, VA; 1:200 dilution in 1% BSA in PBS, 1 h, room temperature). After being rinsed in PBS, sections were immersed in 0.35% hydrogen peroxide in PBS for 10 min to quench endogenous peroxidase activity. The subsequent incubation with biotinylated goat anti-rabbit IgG (1:100; Vector Laboratories) in 1% BSA in PBS lasted 30 min at room temperature, after which slides were incubated with avidin-biotin complex (ABC; Vector Laboratories) during 1 h, according to the manufacturer's protocol. DAB (3,3'-diaminobenzidine tetrahydrochloride, Dako, Glostrup, Denmark) substrate development was done for 30 sec. Nuclei were counterstained with 5% Mayer hematoxylin for 45 sec, and slides were mounted with Pertex (Cellpath Ltd., Hemel Hempstead, UK) after dehydration. For negative control, the primary antibody was replaced by the same concentration of normal rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA).

In Situ Detection of DNA Fragmentation

The in situ terminal deoxynucleotidyl transferase (TdT) mediated by deoxy- UTP-digoxigenin nick-end labeling (TUNEL) method was used to localize apoptotic cells, as described by van Bragt et al. [21]. Sections were counterstained and mounted as described above. For control purposes, the TdT enzyme was excluded from the TUNEL reaction mixture.

To determine whether apoptotic germ cell loss occurs during a specific phase of spermatogenesis, 100 cysts containing apoptotic cells were examined in each of seven males sampled in November that presented complete spermatogenesis (i.e., all germ cell stages present). These 700 cysts (100%) then were examined for the germ cell type they contained, and the incidence of apoptosis was expressed as percentage of cysts containing apoptotic spermatogonia, spermatocytes, or spermatids.

Morphometric Determination of the Number of Spermatogonial Generations

To assess the number of spermatogonial generations, five testes were fixed in November (GSI 5.98 ± 3.1) in 5% glutaraldehyde (Merck), and 5 mM phosphate buffer and were embedded in resin (Technovit 7100; Heraeus Kulzer, Wehrheim, Germany) according to conventional techniques. Serial sections of 3 μm were prepared and stained with 1% toluidine blue containing 1% borax.

To estimate the number of pachytene spermatocytes per cyst, and thereby to conclude how many mitotic divisions the spermatogonia completed before entering meiosis, it was

necessary to determine the average volume of spermatogenic cysts containing pachytene spermatocytes and the average volume of a pachytene spermatocyte. The volume of pachytene spermatocytes was calculated from the nuclear volume and the proportion between nucleus and cytoplasm. To assess the volume of the nucleus, its diameter was measured (30 pachytene spermatocytes per animal; n = 5). Since the pachytene nucleus is round, its volume was estimated using the formula $4 / 3\pi R^3$ (R = diameter / 2), expressed in μm^3. To calculate the proportion between nucleus and cytoplasm, a grid with 121 intersections was placed over the sectioned material at 400 x magnification. For each animal, 1000 points over pachytene spermatocytes were counted.

The cyst volume was estimated using the Cavalieri method of reference volume [22] using serial sections. Only cysts comprised completely in the serial sections were used for evaluation. From the first to the last section of each cyst, the cyst area was measured and multiplied by the thickness of the section, providing the volume of the cyst per section, which when summed up provided the volume of the entire cyst. To measure the cyst area in each section, the Image J analysis program was used (http//:rsb.info.nih.gov/ij/features.html). We analyzed six to eight cysts per animal (n = 5).

RESULTS

Changes in Gonadosomatic Index and Testicular Lobe Composition

Cod testes are paired, longitudinal organs stretching dorsally through the length of the body cavity, connected to the dorsal body wall via the mesorchium. The spermatogenic parenchyma of the cod testis is composed of several lobes of similar morphology that are arranged around and drained by one central efferent duct per testis (Fig. 1). It is a highly dynamic tissue/organ as indicated by the dramatic (41-fold) changes in the gonadosomatic index (GSI; Fig. 2), which ranged from 0.2% (August) to 8.2% (March) on average.

Histological analysis showed that the small, thin, rose-colored lobes of the immature testis (June/July; Fig. 1a) predominantly contained spermatogenic cysts with a single spermatogonium or small groups of spermatogonia; tubules did not yet show a continuous lumen (Fig. 3a). In fully mature males (February to April; Fig. 1c), the large, thick, white lobes were composed of spermatogenic tubules with a large lumen filled with spermatozoa (Fig. 3b). These two stages were connected by a developmental process that established in each lobe a marked gradient of spermatogenic cysts containing germ cells at different stages of maturation. Spermatogenic cysts with more advanced germ cells were situated closer to the central

collecting duct, whereas cysts with germ cells at early stages of spermatogenesis were found in the distal, peripheral part of the lobe. This gradient was particularly evident in samples collected during the rapid growth phase (September to December) and was visible macroscopically (Fig. 1b) and microscopically (Fig. 4a). During this rapid growth phase, the periphery of the lobes was thin, rose colored, and contained mainly different spermatogonial generations (Fig. 4b). The off-white colored, somewhat thicker central area of growing lobes contained predominantly spermatocytes and early (round) spermatids (Fig. 4c), whereas the white area close to the collecting duct contained late (elongated) spermatids and spermatozoa (Fig. 4d). With further increase of testis weight and GSI, the white area made up an increasing proportion of the growing lobe, eventually occupying the complete lobe in a whitening wave that emanated from the central collecting duct.

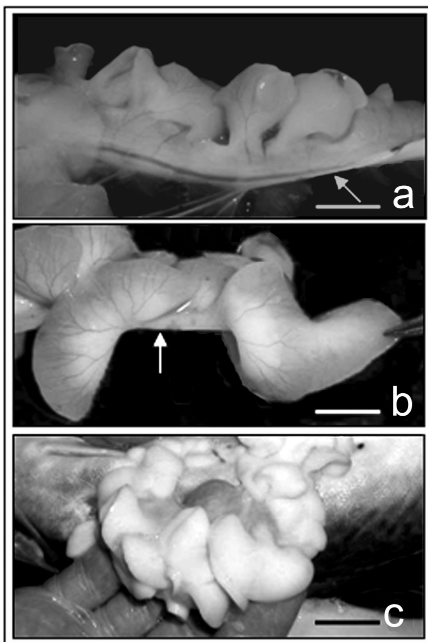

Figure. 1. Macroscopic view of: (**a**) immature, (**b**) maturing, and (**c**) fully mature cod testis. Arrows indicate efferent duct (obscured by large lobes in **c**). In **b**, a gradient of color can be observed from gray at the lobe's periphery, to white in direction of the efferent duct. Bars = (**a**) 6 mm; (**b**) 10 mm; and (**c**) 15 mm.

Histologically, this was reflected in an increasing proportion of the tubules being filled with mature spermatozoa, whereas the more peripheral zone of spermatocytes and spermatogonia became progressively smaller, until all spermatogenic cysts had reached the spermiation stage (cysts open and Sertoli cells releasing spermatozoa into the tubular lumen), and the entire testis lobe was filled with sperm. With the spawning-associated release of sperm, the GSI started to decrease (April). Low values were attained already in June in spent testes, when early spermatogonia were the only germ cells present next to residual sperm being progressively removed by Sertoli cells via phagocytosis (Fig. 3, c and d). Cysts were no longer

observed in the testis lobes, and Sertoli cells formed an epithelial layer lining the tubular lumen (Fig. 3d). Figure 5 schematizes the developmental changes in a testicular lobe, highlighting its marked gradient of maturation.

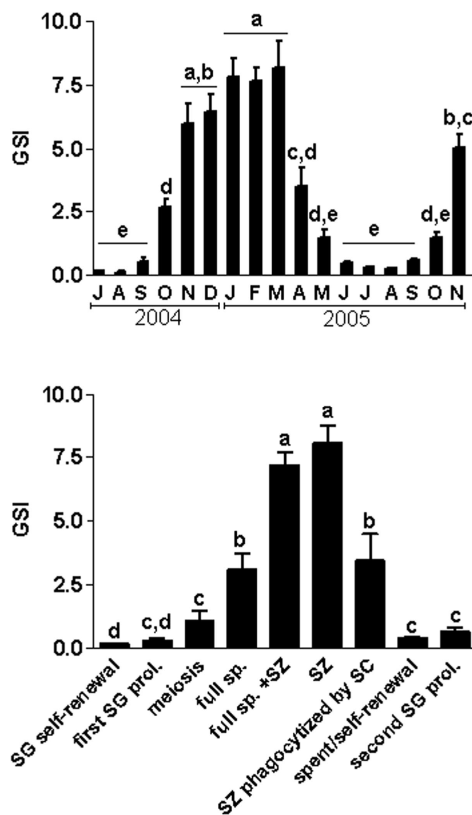

Figure. 2. **a**) GSI values per month in male Atlantic cod starting with July (indicated with a ''J''). Measurements are from July 2004 to November 2005 (n = 10–18). Values of GSI are indicated in the y axis (%; mean ± SD). **b**) GSI values according to histologic characterization of the testes (n = 7–23 per group). Different letters denote significant differences, $P < 0.05$. SG, spermatogonia; SZ, spermatozoa; SC, Sertoli cell; prol., proliferation; sp., spermatogenesis.

Spermatogonial Generations and Analysis of Spermatogenesis

The average volume of a pachytene spermatocyte was 70 ± 2 μm^3 per cell. Analyzing the volume of a total of 37 pachytene spermatocyte cysts, we found that two thirds of the cysts showed a volume indicating that the spermatogonia went through 11 mitotic cell cycles, whereas the volume of the remaining one third indicated that 12 mitotic cell cycles were completed. These data resulted in an average volume of pachytene spermatocyte cysts of 136 ± 25 mm^3, suggesting that approximately 1900 pachytene spermatocytes were present per cyst. We therefore conclude that at least 11 mitotic cell cycles are completed before entering meiosis (n.b. $2^{11} = 2048$).

To evaluate further the mitotic phase of spermatogenesis, we analyzed sections after immunocytochemical detection of phosphorylated histone H3, a mitosis marker. Generally, the massive spermatogonial proliferation started in August when, besides single spermatogonia, the first cysts of late spermatogonia were observed proliferating through the lobe (Fig. 4e). Sertoli cells proliferated as well (Fig. 3a, inset), and their mitotic activity was observed mainly

while they were associated with early spermatogonia. In October, the end of spermiogenesis was attained in the furthest progressed cysts in some of the males, and spermiation started (i.e., the cysts opened and spermatozoa were released in the tubular lumen). Free sperm was present in tubules near the collecting duct, but cysts with meiotic and postmeiotic germ cells as well as cysts still containing proliferating spermatogonia were found in other areas of the lobes (Fig. 4, e–g). Strong spermatogonial proliferation activity and formation of new spermatogonial cysts in the periphery of the lobes were observed until January. From February onward, however, formation of cysts with proliferating spermatogonia had stopped, and most cysts had entered meiosis so that proliferating spermatogonia were found only rarely in the testis and moreover did not form developing cysts, but were single or paired spermatogonia.

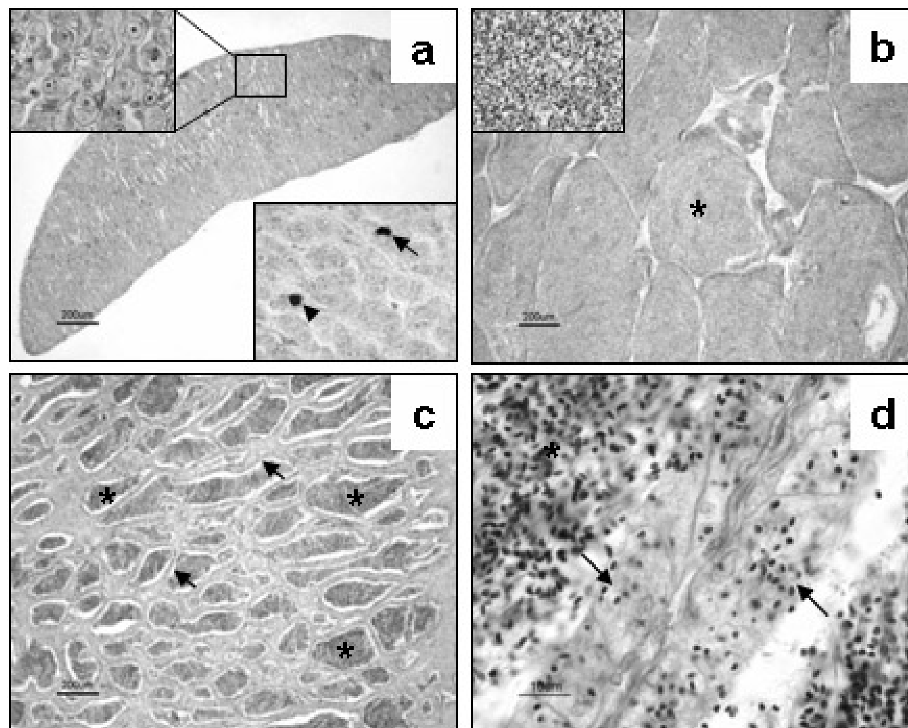

Figure. 3. Histology of cod testis in different stages of development. **a**) Immature; inset top left shows that early spermatogonia are the predominant germ cell type present at this stage; inset lower right shows an early spermatogonium (arrowhead) and a Sertoli cell (arrow) proliferating, as shown by pH3 immunolabeling. **b**) fully mature testis; asterisk shows seminiferous tubule filled with free spermatozoa. **c**, **d**) Spent testis; asterisk shows seminiferous tubule filled with free spermatozoa; arrows indicate phagocytized spermatozoa inside the Sertoli cell cytoplasm.

Apoptosis

TUNEL analysis indicated that germ cell death did not occur frequently in cod spermatogenesis, since cysts presenting positive germ cells were rare. The highest incidence

was observed during the last stages of spermatogonial proliferation, possibly just before entering meiosis (Fig. 4, i and j), involving 72% of the apoptotic germ cells, followed by spermatocytes and spermatids, which represented 12% and 11%, respectively; 5% remained nonidentified due to the lack of clear cytologic features when cells were far progressed into apoptosis. Germ cells in apoptosis showed the expected nuclear staining, reflecting the DNA fragmentation, but in some cysts the Sertoli cell cytoplasm was also labeled with the TUNEL technique (Fig. 4i).

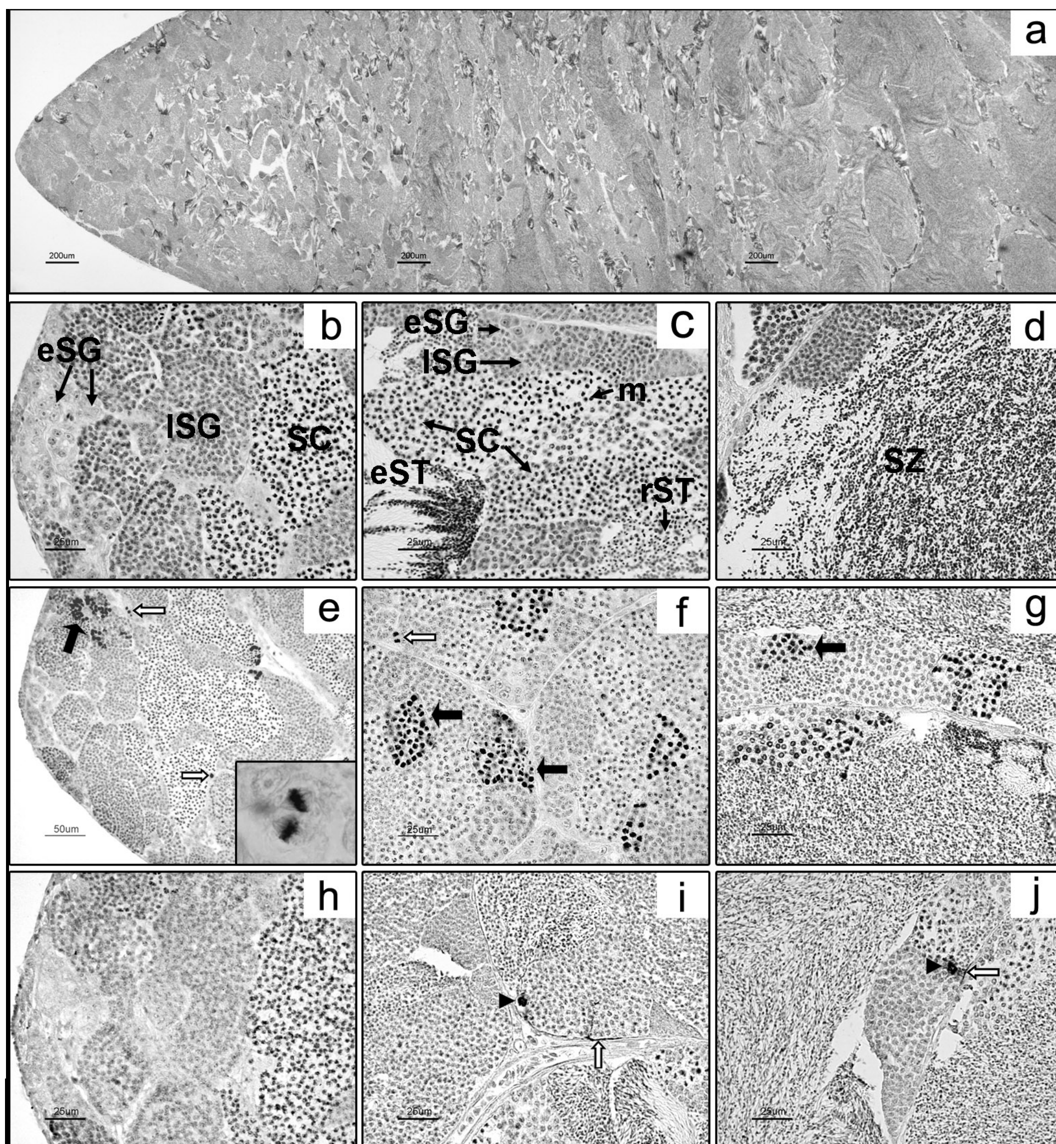

Figure. 4. Different areas of a cod testis lobe during spermatogenesis, representing the periphery (**b**, **e**, **h**), the central area (**c**, **f**, **i**), and the area close to the collecting duct (d, g, j). a) Histologic overview of one lobe. **b**–**d**) hematoxylin-eosin staining. **e**–**g**) Immunohistochemistry for pH3. **h**–**j**) TUNEL labeling. eSG, early spermatogonia; lSG, late spermatogonia; SC, spermatocytes; rST, round spermatids; eST, elongated spermatids; SZ, spermatozoa; m, meiotic figure. **e**–**g**) arrows indicate cells positive for pH3, early (white) and late (black) spermatogonia; inset in e shows a five times higher magnification of labeled spermatogonia in metaphase. **h**–**j**) arrowheads indicate TUNEL-positive germ cells, and white arrows indicate Sertoli cell cytoplasm. No TUNEL positive cells are present in the lobes periphery in **h** due to the absence of spermatogonia just before entering meiosis.

DISCUSSION

The initiation of spermatogenesis marks the onset of puberty, which in cod is associated with an impressive growth of the testes. The GSI started to increase in September, reaching more than 8% of the total body weight in fully mature fish. Similar GSI values were observed in cod either captured in Canada (9%) [23, 24] or reared in Norway (10%) [25]. Higher maximum GSI values of up to 17% were found in a previous study with cod that were 1 yr older [26].

Testicular weight gain is attributable mainly to germ cell proliferation during spermatogenesis. Indeed, the variation in GSI values in cod testes correlated with the observed changes in testis histology. During periods with low GSI values (prepubertal-immature and postspawning-spent testes), the spermatogenic parenchyma showed only early spermatogonia and Sertoli cells in the germinal epithelium, while a lumen had not formed yet. The first elevation in GSI reflected the initiation of the mitotic activity of these two cell types. Hence, starting in August, proliferating spermatogonia were observed regularly until January and continued their development toward meiosis and spermiogenesis, so that spermatogenic cysts increased progressively both in number and size along with the GSI. Decreasing GSI values were observed after the end of spermatogenic activity and during the course of the spawning period, reflecting the release of sperm. The lowest GSI values were attained in June/July, reflecting completion of the removal of residual sperm via Sertoli cell phagocytosis, whereas the new spermatogenic wave had not started yet.

A very interesting feature of cod spermatogenesis is the maturational gradient in which spermatogenic cysts become organized during testis growth. This gradient of progressively mature germ cells is established in each lobe, suggesting that a spatiotemporal regulation of the lobe's histo-architecture is implemented throughout the testis. The origin and driving force of the gradient seems to be the peripheral rim of the lobe, where new cysts with proliferating spermatogonia are formed and which is therefore referred to as the *germinative zone*. The addition of new spermatogonial cysts results in appositional growth of the lobes and displacement of the germinative zone from the collecting duct. Further growth is achieved by the increasing cell number of the developing cysts while procession through spermatogenesis. The newly formed cysts do not appear to move during their growth/maturation while new cysts are being added from the lobe's peripheral germinative zone. The latter ceases in late January, as reflected by the disappearance of large cysts with spermatogonial cells positive for pH3. Consequently, since spermatogenesis continues at a predictable speed [27], all cysts reach the spermiation stage, and the tubules' lumina become filled with spermatozoa in a

maturational wave proceeding from the central collecting duct area towards the periphery of the lobes. An akin pattern of spermiation (spermatozoa being released first from cysts near the collecting duct) was observed in the common snook (*Centropomus undecimalis*) [28]. The marked spatiotemporal organization of cod spermatogenesis in conjunction with the lobular composition of the spermatogenic parenchyma has not been described in other teleost fish, and it may represent a good model to study the activity of locally active factors controlling the spermatogenic process. For instance, it will be very interesting to study 1) what factors are responsible for the start of cyst formation in the germinative zone in August and its cessation in January, 2) how these hypothetical factors are regulated by the brain-pituitary system, and 3) how external factors (e.g., photoperiod) that are known to affect pubertal maturation [25] would modulate the regulation via the brain-pituitary system.

The present study is the first one describing in detail testis morphology and the histologic organization of spermatogenesis in a species from the order *gadiformes*, and introduces the concept of a maturational gradient in testicular lobes. The impressive overall growth of the testis represents the combined growth of several individual lobes that seem to function as independent units. Since cod are fish showing continuous growth during adult life, we anticipate that further growth of the testes in subsequent years would be realized via a caudal extension of the collecting ducts and the outgrowth of additional lobes. In salmonid fish, testis tissue also undergoes several-fold weight changes during spermatogenesis, but the spermatogenic parenchyma is organized in two compact organs and there is no predictable distribution of spermatogonial cell types along a maturational gradient [29]. Flatfish of the genus *Solea* do show testes where spermatogonia are located preferentially in the testicular periphery, but the testes are small and the increase in GSI during maturation of the sole testis is always very low [30].

In cod testis, all spermatids present in one cyst developed synchronously and presented the same shape and nuclear condensation. This is different from the situation in rainbow trout as described by Billard [31], who recorded certain heterogeneity during spermiogenesis within one cyst. Following spermiation, free spermatozoa were present in the gonad for a long period, from January to May, as previously described [16, 24]. After the spawning season, that is, from June, spermatogenic cysts in development were absent, and Sertoli cells phagocytized residual spermatozoa, then becoming more vesiculated. In some animals, spermatozoa were found in the testis up until the beginning of the second spermatogenic cycle, and they seemed to be eliminated later by Sertoli cells rather then being stored for the next season.

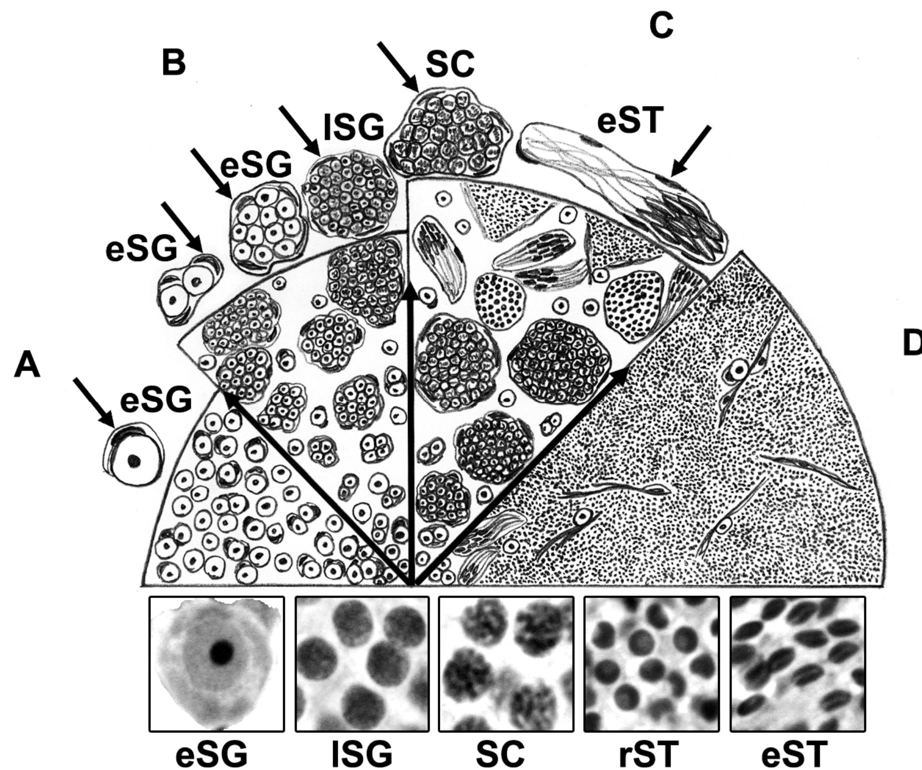

Figure. 5. Drawing representing a cod testis lobe during development of spermatogenesis. **a**) immature testis; **b**) testis during spermatogonial (and Sertoli cell) proliferation, when the first spermatogenic cysts can be observed; **c**) testis through spermatogenesis; and **d**) testis after Spermiation (mature). Arrows indicate Sertoli cells. The arrows present between the schematic lobes indicate the disposition of the lobe, growing from the apex to the collecting duct area. eSG, early spermatogonia; lSG, late spermatogonia; SC, spermatocytes; rST, round spermatids; eST, elongated spermatids.

Although early spermatogonia were found preferentially in the peripheral rim of the testis lobes (germinative zone), some were also found dispersed throughout the epithelium of the seminiferous tubules, located as single cells between cysts of further advanced stages of spermatogenesis, a feature cod share with other teleosts [26, 28, 32, 33]. In our studies on proliferation, we observed that these cells were only rarely active, suggesting that these cells are quiescent and represent a reserve population.

Generally, the number of mitotic divisions of spermatogonia preceding meiosis is species specific in vertebrates [7, 34, 35]. In different teleosts, it has been found that spermatogonia divide 5 to 14 times before differentiating in spermatocytes [7, 11, 12, 33, 36–38]. The two known methods used for estimating the number of spermatogonial generations, counting the number of germ cells or measuring cyst diameter [7, 12], could not be used in cod testis. The former because the number of germ cells per cyst was too high, decreasing the confidence of the methodology, and the latter because the cysts in cod were irregularly shaped. Hence, an alternative method was developed for this study. It consisted of determining the average volume occupied by a cyst with pachytene spermatocytes, which was then divided by the average volume of a pachytene spermatocyte. With this approach, the number of mitotic

divisions that spermatogonia undergo in cod was determined to be 11, which is among the highest found in vertebrates. The small difference between the theoretical (2048) and measured (1942) number of primary spermatocytes is in line with the very low incidence of apoptosis, implying that cod spermatogenesis, similar to the guppy *Poecilia reticulata* [11] or the zebrafish *Danio rerio* [39], is rather efficient among teleost fish, which in general seems more efficient than mammalian spermatogenesis [40, 41].

During the spawning season, female cod spawn 10–20 batches of eggs, with a new batch produced every 2–3 days [42]. This requires repeated mating and, therefore, a large number of spermatozoa for the external mode of fertilization. One reason that may contribute to the high spermatogenic efficiency in cod (and other fishes) is Sertoli cell proliferation, which typically accompanies the spermatogonial phase of cystic [5], in contrast with avian or mammalian, spermatogenesis. Hence, the number of Sertoli cells is adjusted to the optimum required during cystic spermatogenesis. In cod testis, Sertoli cell proliferation was observed by histone H3 phosphorylation immunohistochemistry. Although not studied systematically here, Sertoli cell proliferation was recorded mainly when these cells were associated with the first spermatogonial generations (i.e., in the periphery of the lobe, rather than when associated with meiotic or postmeiotic stages). This suggests that the germinative zone is an environment stimulating or allowing Sertoli cell proliferation.

As already mentioned, germ cell loss in cod spermatogenesis was relatively rare. However, when it occurred, it was more common during the late spermatogonial phase, particularly during the last mitotic divisions prior to spermatocyte formation. The degeneration of spermatogenic cells at the transition stage from spermatogonia to spermatocytes is frequently observed in seasonally breeding animals [43], although in some teleost fish the apoptotic incidence is highest during spermiogenesis [5, 11, 12]. Also, in the phylogenetically older dogfish (*Squalus acanhtias*), germ cell degeneration occurs during the differentiation to spermatocytes [44, 45], whereas this occurs during the meiotic phase in the spotted ray [46]. Interestingly, not all germ cells of a given cyst went into apoptosis, but only one to five spermatogonia in a given cyst. This situation is normally described in the literature, despite the intercellular bridges between cells originated from the same clone [47], and it indicates that it is rather a developmental problem of a specific germ cell than a problem of the complete clone or of the cyst-forming Sertoli cells. In the present study, besides the expected nuclear labeling of germ cells, Sertoli cells near apoptotic germ cells sometimes also presented TUNEL staining that was, however, a cytoplasmic staining. The latter might reflect an important and well-known task of Sertoli cells, the removal of apoptotic germ cells by phagocytosis.

In summary, cyst development occurs in a specific spatiotemporal organization during cod spermatogenesis, leading to an appositional growth of testis lobes. With 11 spermatogonial mitotic divisions and a high number of germ cells per cyst, combined with a low incidence of apoptosis, spermatogenesis in cod is one of the most efficient among teleost.

ACKNOWLEDGMENT

The help from Fritz Kindt with composing the histology plates is highly appreciated.

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Photoperiod-Modulated Testis Maturation in Atlantic Cod (*Gadus morhua*, L.)

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Biol Reprod, April 2009; 80: 631 - 640.

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ABSTRACT

Precocious male puberty is a significant problem in Atlantic cod aquaculture. While photoperiod manipulation can inhibit testis growth, a detailed analysis of effects on spermatogenesis is missing. Starting July 1, 2004, prepubertal fish were exposed to different photoperiod regimens in indoor tanks for 17 mo. Testis histology, germ cell dynamics (proliferation and apoptosis), and plasma androgen levels were analyzed. In the natural light (NL) group, testis growth started in September 2004 and was completed in February 2005, when a 2-mo spawning period started. In the constant light (LL) group, none or very few spermatogenic cysts were recruited into spermatogenesis, and apoptotic germ cell loss was high. A change of photoperiod from NL to LL at winter solstice (December 21, 2004) resulted in premature (2 mo) completion of the reproductive cycle, while changing from LL to NL at winter solstice triggered faster than normal testis development. Plasma testosterone levels increased in the NL group from spermatogonial proliferation toward meiosis, while those of 11-ketotestosterone increased toward spermiogenesis and spermiation. Plasma androgen levels did not rise under LL conditions. Comparing fish with developing testes from all groups indicated that low androgen levels were associated with a high incidence of spermatogonial apoptosis; we also found that androgen receptor mRNA expression was most prominent in Sertoli cells in contact with growing spermatogonial clones. Our data show that an inhibitory photoperiod (LL) reduced or blocked differentiation of spermatogonia, increased apoptosis (particularly among proliferating spermatogonia), and was associated with reduced androgen levels, a situation possibly reflecting insufficient gonadotropic stimulation.

INTRODUCTION

In recent years, there has been a rapid development of Atlantic cod (*Gadus morhua* L.) farming in the North Atlantic, as a response to declining wild stocks. Although its potential for farming is considerable, farmed male Atlantic cod enter puberty earlier than wild specimen. Wild cod mature at an average age of five years [1] while under the optimal growth conditions in captivity the first gonad maturation can be observed in one and two year old fish [2, 3]. This

early maturation reduces appetite [4], causes weight loss [2], restricts growth [5], and decreases flesh quality at the end of the spawning season [6], features compromising the sustainability of cod aquaculture [7]. The physiological background of early male puberty has not been characterized.

During spermatogenesis, stem cells produce spermatogonia that proliferate mitotically to increase germ cell number, before entering meiosis and spermiogenesis to give rise to haploid, flagellated spermatozoa [8]. In fish (and amphibians), spermatogenesis takes place within cysts formed by Sertoli cells that envelope a developing germ cell clone derived from one spermatogonial stem cell [9]. The spermatogenic cysts are situated in the seminiferous tubules and together constitute the spermatogenic epithelium of the testis. In Atlantic cod, the testis is composed of several lobes that insert into a central, common sperm duct. Cysts with spermatogonia are located in the periphery of these lobes, while more mature stages are found closer to the sperm duct, hence establishing a gradient of cellular maturation in each lobe [10, 11].

Photoperiod has a major effect on the activity of the brain-pituitary axis, and consequently on gonad maturation and activity in vertebrates [12, 13]. In teleost fish, photoperiod cues can modulate maturation [14 – 17] and therefore have been tested as means to delay or inhibit early male puberty during the ongrowth period in cod [5, 18-20]. In these studies, spawning activity and/or plasma steroid levels were recorded, and gonad growth was assessed using ultrasound or by determining gonadosomatic indeces (GSI) or oocyte diameters. No detailed information is available regarding the effects of photoperiod manipulation on the complete process of spermatogenesis and thus it is not known which phase(s) of spermatogenesis (spermatogonial proliferation, meiosis, spermiogenesis, sperm storage) is/are photoperiod-sensitive.

It is reasonable to assume that environmental cues, such as photoperiod and temperature, are integrated by the brain, subsequently triggering changes in the activity of physiological systems regulating spermatogenesis. Hence, the brain-pituitary axis would produce endocrine signals mediating these environmental signals to the testis. Sertoli cells are an important target for the endocrine regulation of spermatogenesis. For example, the Sertoli cell-specific loss of androgen receptor (Ar) function results in complete spermatogenic failure in mice [21]. In eel, as in other fishes, the follicle-stimulating hormone (Fsh) triggers the production of androgens [22, 23], which in turn stimulate spermatogonial proliferation and differentiation and meiosis via Sertoli cell activation [24]. Two types of androgens are important for regulating spermatogenesis in teleosts, testosterone (T) that is known for its feedback effects on the brain-pituitary axis [25, 26] and 11-ketotestosterone (11-KT), an androgen typically

found in fish that stimulates spermatogenesis, secondary sexual characteristics, and sexual behavior [27, 28].

In this study, we aimed at identifying the phase(s) of spermatogenesis that is(are) modulated by the photoperiod. Using combinations of photoperiod regimes, we created four experimental conditions that resulted in largely different testicular growth patterns. These patterns have been analyzed morphologically (testis histology, germ cell proliferation and apoptosis) and put into context with plasma androgen (T and 11-KT) levels in a large number of individuals.

MATERIAL AND METHODS

Animals, Light Treatment Groups and Sampling

Male and female prepubertal Atlantic cod (Norwegian Coastal cod) were reared in 3-m-diameter, 1-m-water height (7 m^3) seawater outdoor tanks at the Institute of Marine Research, Austevoll Research Station, Storebø, Norway (60^0 north). The larvae hatched in March 2003 and were first fed natural zooplankton in a semienclosed seawater pond before transfer to the experimental tanks, where they were fed a commercial dry pellet diet (DanEx 15–62; Danafeed, Horsens, Denmark) *ad libitum*. The tanks were supplied with seawater pumped from 168 m depth, and the mean ± SD water temperature ranged from 7.4 ± 8.1^{0}C to 9.4 ± 0.3^{0}C during the experimental period. All fish were treated and euthanized according to Norwegian National Legislation for Laboratory Animals [29].

From July to December 2004, the animals were divided into two groups, each in six replicate tanks (initial n = 175/tank). One group was reared under natural light (NL), where the tanks were covered with 70% light-reducing shading nets, and the other group was exposed to LL in light-proof tanks. The LL light was supplied by 70 W metal halide lamps (MHW-TD 70W light bulbs; Phillips, Amsterdam, the Netherlands), giving an integrated irradiance of 5.5 e^{-4} W sec^{-1} cm^{-2} just below water surface. Starting December 21, 2004, three tanks of each group were exposed to the other light condition until November 2005, resulting in four experimental groups: 1) NL or 2) LL conditions during the complete experimental period (17 mo), 3) 6 mo of NL followed by 11 mo of LL (NL→LL), or 4) 6 mo of LL followed by 11 mo of NL (LL→NL). Each treatment was performed in triplicate tanks. Every month, 30 fish were randomly sampled from each treatment. The first 6 mo (July through December 2004), five fish were sampled from each of the six tanks exposed to NL or to LL. From January through November 2005, 10 fish were sampled from each of the three tanks exposed to NL, LL, NL→LL, or LL→NL. There were

no statistically significant differences in GSI between animals from the six or three replicate tanks from the same light treatment, so that all data were pooled according to the photoperiod regimen. The mortality was low, with an overall loss of 9% during the 17 mo period. All 60 or 120 fish/mo were sampled on the same day and always in the same sequence of tanks, with a random distribution of the replicates during the sampling day.

At the start of the experiment (July 1, 2004), the fish were prepubertal and 18 mo of age. They weighed on average 807 g, 1750 g in December 2004, and 3391 g at the termination of the experiment in November 2005. The sex ratio was 50% (integrated for all the samplings); testis and blood samples were collected monthly from 10 to 19 males (i.e., there were 11–20 females). After blood sampling, body weight and testis weight were recorded to calculate the gonadosomatic index (GSI = testis weight X 100 / body weight). Testis tissue was fixed in Bouin solution, dehydrated, and embedded in paraffin according to conventional techniques. For morphological analysis, 4 µm thick sections were stained with hematoxylin-eosin (HE). The presence of running milt in the efferent duct system was noted as ‘‘spawning’’ during sampling when slight pressure on the abdomen induced milt release from the genital pore.

Plasma androgen levels were quantified in all animals. The number of males analyzed per treatment group and the sampling date for proliferation (the same number of samples was analyzed histologically after HE staining) or apoptosis are given in Supplemental Table 1.

Morphometric Determination of the Number of Spermatogonial Generations

The analysis was restricted to the LL-exposed group that contained a subset (35%) of fish showing spermatogenic activity with comparatively small cysts and low GSI values. To investigate if small cysts and low GSI values were related to proceeding through a lower than normal number of spermatogonial mitoses, the number of mitotic cell cycles was determined as described previously [11] and compared to the previously determined results from the NL group of this experiment. To this end, tissue samples were fixed in 5% buffered glutaraldehyde (Merck) and embedded in resin (Technovit 7100, Heraeus Kulzer, Wehrheim, Germany), according to conventional techniques. Four to eight cysts were analyzed per animal from 3 males of the LL group showing spermatogenic activity (randomly selected from November 2004).

Proliferation and Apoptosis

Proliferation of spermatogonia and Sertoli cells was assessed by phosphorylated histone H3 (pH3) immunodetection, as described previously [11]. The protein becomes phosphorylated

during the late G2 phase of the cell cycle and remains detectable until the metaphase [30], i.e. is present in cells preparing to divide.

The TUNEL method was used to localize apoptotic cells. This has been described previously [11].

The maturation- and photoperiod-induced changes were very clear and individuals either showed high or low proliferation activity/apoptosis; intermediate levels were not found (see supplemental Figs. 1 and 2). For example, proliferation activity was low before but high after the start of spermatogenesis in the NL group (supplemental Fig. 1 a and b, respectively), or low in LL-inhibited fish (supplemental Fig. 1 e) but high after transfer to NL (supplemental Fig 1 g - i). Also as regards apoptosis, the incidence was either low (e.g. in all NL testes and all fish with quiescent testis), or high (e.g. LL testes with spermatogenic activity and testes after transfer from LL->NL during rapid growth phase and low androgen plasma levels) (supplemental Fig. 2). Therefore, quantification of the observations on proliferation and apoptosis was considered unnecessary.

***In situ* hybridization**

The full-length open reading frame of the cod androgen receptor was amplified, cloned into the pcDNA3.1/V5-His TOPO vector (Invitrogen, San Diego, CA), and sequenced (GenBank accession number FJ268742; our unpublished results). Then, specific primers (Forward: nucleotides 19–46: 5′- GGGCGGGTG*TTATTAACCCTCACTAA*AGGGTAAGCC AGTCCCCTCTTGTCGGAAAA- 3′, with the T3 RNA polymerase promoter in italics; Reverse: nucleotides 499–519: 5′-CCGGGGGGTG*TAATACGACTCACTATA*GGGATGAGCACGCGTCT CGGG- 3′, with the T7 RNA polymerase promoter in italics) were designed to PCR amplify a cod androgen receptor cDNA fragment (~ 480 bp) for sense and antisense digoxigenin-labeled cRNA probe synthesis using T3 and T7 RNA polymerase, respectively.

Cod testes were collected for in situ hybridization from animals exposed to NL conditions during the rapid growth period in the fall of 2007. Histological analysis showed that proliferating spermatogonia, spermatocytes, and spermatids were present, while spermiation had not occurred yet. The tissue was fixed in 4% w/v paraformaldehyde in PBS, immersed in 20% sucrose at 4^0C overnight, frozen in Tissue Tek (Sakura Finetek Europe B.V., Zoeterwoude, the Netherlands), and stored at -80^0C until use. Cryosections of 10-µm thickness were cut at -20^0C and mounted on Super-Frost slides (Menzel-Glaser, Braunschweig, Germany). *In situ* hybridization was performed as described by Weltzien and colleagues [31] using sense and antisense cRNA probes at a concentration of 400 ng/ml of hybridization buffer. The

ribonuclease A (15 µg/ml; Sigma-Aldrich, Steinheim, Germany) treatment was performed in RNase buffer (0.01 M Tris, 0.5 M NaCl, 0.005 M editic acid [EDTA], pH 7.5).

Plasma Androgen Assays

Steroids were extracted from plasma samples as described by Hyllner et al. [32], dissolved in 1 ml of buffer (0.1M phosphate pH 7.4, 0.4M NaCl, 1mM EDTA, 0.1% BSA) by heating (60^0C; 10 min.) and stored at -20^0C until analysis by enzyme-linked immunosorbent assay (ELISA) [33]. Steroid standards were purchased at Sigma-Aldrich. Antisera against testosterone (T) and 11-ketotestosterone (11-KT) were kindly provided by Dr. Silvia Zanuy (CSIC, Spain) and Dr. David E. Kime (University of Sheffield, UK), respectively. Coated microtiter plates (Mouse-anti-Rabbit) were purchased from SPI-BIO (France).

Details on cross-reactivity of the antisera against T and 11-KT were given by Rodriguez et al. [34] and Cuisset et al. [33], respectively. The lower limits of detection were 10 pg/ml for T (ED80) and 20 pg/ml for 11-KT (ED90). Cod plasma dilutions were parallel to the standard curves. The inter-assay coefficients of variation for the extracted biological reference plasma were 13.5% for T (n = 33) and 10.9% for 11-KT (n = 20). The intra-assay coefficients of variation were 5.6% for T and 8.3% for 11-KT.

Statistics

First, we tested for differences in GSI (of males) between tanks subjected to the same photoperiod regime during all the experiment period. No significant differences were found between tanks, and data were pooled according to the photoperiod regime. Data are presented as means ± SEM. Statistical differences of GSI and androgen levels were analyzed by one way ANOVA followed by Student-Newman-Keuls multiple comparison test with a significance level (p) of 0.05. For GSI, 11-KT and T the monthly data were analyzed within (i.e. change over time) and between (i.e. change depending on photoperiod treatment) groups. For analyzing the number of spermatocytes per cyst, the Student t test was used. To achieve homogeneity of variance, plasma androgen values were $\log_{10}$-transformed; GSI values were arcsin-transformed.

To analyze a possible relation between sex steroid levels and apoptosis, we first selected individuals that showed spermatogenetic activity, i.e. we excluded animals with quiescent and with fully mature testis (both showing very little apoptosis). This subset (51 males from all groups) was further differentiated into 23 males during rapid spermatogonial proliferation just after the start of spermatogenesis, and 28 males where spermatogenesis had progressed into meiosis/spermiogenesis. Then, we grouped these animals according to the incidence of

apoptosis, before comparing androgen plasma levels in animals with a low (e.g. Fig. 3 a or d) vs. a high incidence of apoptosis (e.g. Fig. 3 b or c).

RESULTS

Spermatogenesis and GSI

In July 2004, all animals presented quiescent testes, i.e. only a few single and/or paired spermatogonia were proliferating. The development under NL conditions has been described previously as regards GSI and histology [11], but is summarized briefly here since photoperiod treatment groups are compared to the NL (control) group. In August, spermatogonia and Sertoli cells started proliferating. In September, many large cysts of replicating spermatogonia were observed. Meiosis and spermiogenesis took place from October to January, the last month that spermatogonial proliferation was observed. Maximum GSI levels were reached in March (Fig. 1a). The cysts formed a gradient of maturation from the germinative zone (early spermatogonia in the periphery of each lobe) to the most developed cysts close to the collecting duct [11]. Spermiation, i.e. opening of cysts to release spermatozoa into the tubule lumen was first observed in November 2004 and was completed in February 2005. Spawning was observed regularly from February to April, when GSI values started to decrease, and when Sertoli cells started phagocytosing spermatozoa. Removal of residual sperm was completed in July, when GSI values reached minimum values. The second reproductive season started in September with spermatogonial proliferation (Fig. 1a).

Under constant light (LL) conditions, the following deviations from the NL pattern were recorded. First, in 50 of 77 males, mitotic germ cell proliferation was extremely low and no maturing cysts were found. These testes were characterized as quiescent (compare Fig. 2a and d) and were found at all sampling dates in the LL group, although less often during the second summer/fall period (Fig 1b). Second, in the 27 males that did show spermatogenic activity, only a limited number of spermatogonial cysts were recruited into mitotic proliferation and meiosis (Fig. 2b). Accordingly, the GSI did not surpass an average of 2.2 ± 0.76% (Fig. 1b). Third, the gradient of maturation from the lobes' periphery towards the collecting duct was absent in LL-exposed males that presented spermatogenic activity. Instead, the few developing cysts were randomly distributed throughout the lobe, independent of the developmental stage of their germ cells (Fig. 2b and c). There was no significant difference between quiescent and partially spermatogenic developing males with regard to body length or weight (data not shown).

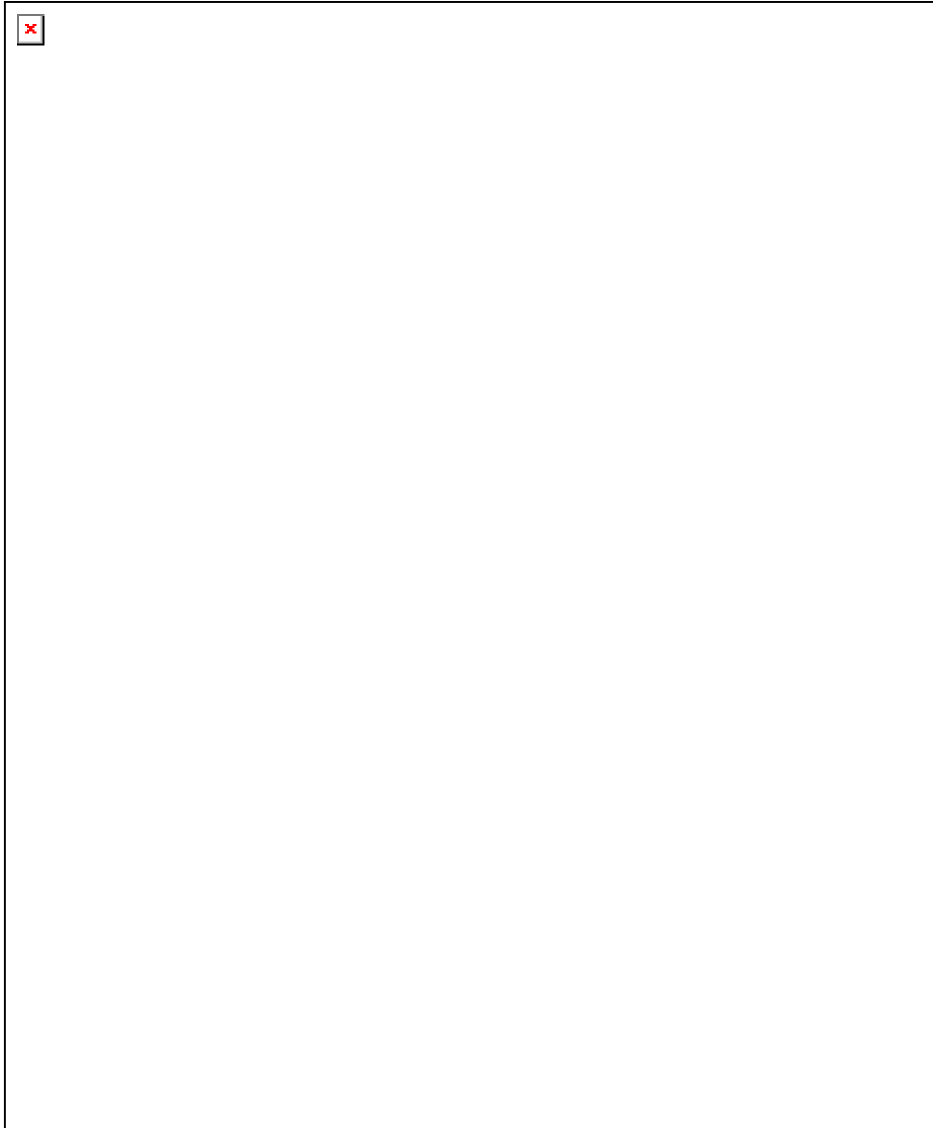

Figure 1: Gonadosomatic index (GSI; solid line), photoperiod regime (hours of daylight; stippled line) and progress through spermatogenesis in male Atlantic cod. Measurements are from July 2004 to November 2005 (n = 10 - 19); **a**) NL: normal light; **b**) LL: continuous light; **c**) NL→LL: changed from normal light to continuous light; **d**) LL→NL: changed from continuous light to normal light. Values of GSI are indicated on the right ordinate (%; mean ± SEM); hours of light are given on the left ordinate. The arrow in **c**) and **d**) indicates the timing of change of the light regime. The numbers in (**b**) and (**c**) give the number of males showing testis with some spermatogenic activity (upper number) and quiescent testis (lower number). Monthly means labeled with the same letter do not differ significantly ($P > 0.05$) within the same treatment group. In (**e**), treatment groups labeled with different letters in the same column (month) differ significantly ($P < 0.05$).

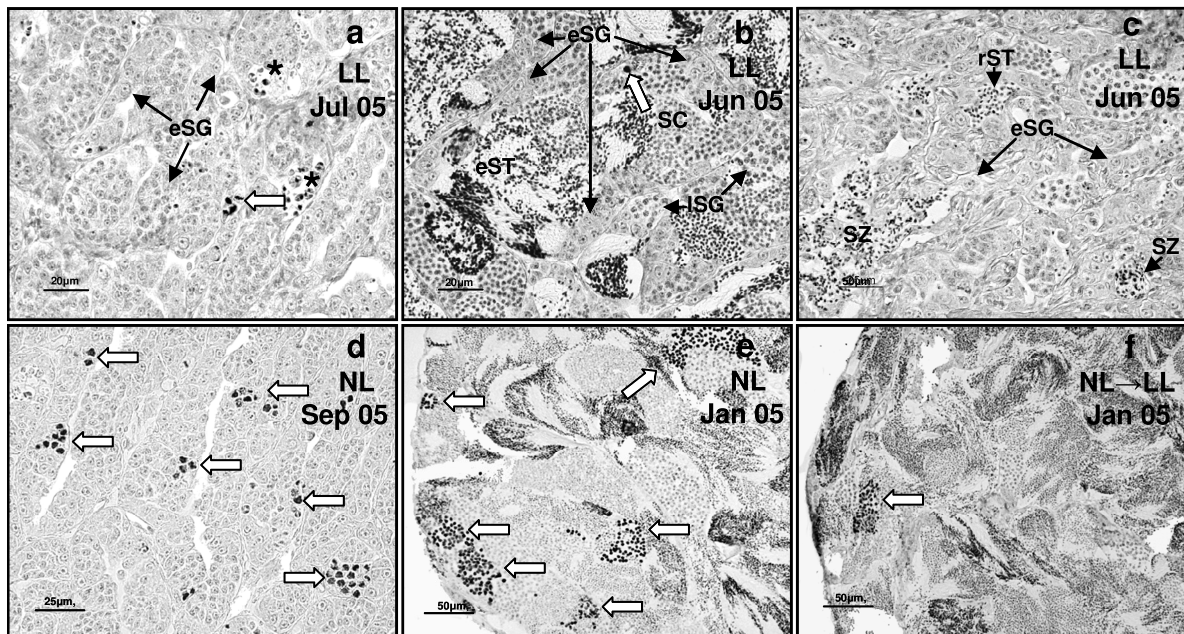

Figure 2: Immunohistochemistry for phosphorylated histone H3 (pH3) in cod testes; **a**) quiescent LL testis (50 of 77 LL males): pH3-positive spermatogonia are rarely observed; **b**) and **c**) LL testis showing spermatogenic activity (27 of 77 LL males); note the extended areas with non-proliferating, early spermatogonia and the random distribution of developing cysts, so that a gradient of maturation is not established; **d**) NL testis in spermatogonial proliferation; **e**) apex of a NL testis lobe in January, showing numerous cysts with proliferating spermatogonia; **f**) apex of NL→LL testis lobe in January, i.e. one month after the shift of the light regime, showing a clear reduction in the number of cysts with proliferating spermatogonia. **eSG**: early spermatogonia; **lSG**: late spermatogonia; **SC**: spermatocytes; **rST**: round spermatids; **eST**: elongated spermatids; **SZ**: spermatozoa. White arrows point germ cells positive for pH3; asterisk: apoptotic cells.

When NL males were moved to continuous light on December 21 (NL→LL), no, or only very few, cysts of proliferating spermatogonia were found in January while they were still abundant in January in testes of the NL group (compare Fig. 2e and f). The GSI values decreased rapidly in the NL→LL group (Fig. 1c; February and March 2005), reflecting the completion of spermiogenesis and, possibly, the release of spermatozoa, while animals in the control group kept high GSI values up to March. Phagocytosis of spermatozoa by Sertoli cells was noted already in March in the NL→LL group, i.e. one month earlier than in the NL group. From April onwards, the males in the NL→LL group showed testes similar to those found in the LL group: some animals (13 of 25) showed quiescent testes, while in others a limited number of cysts proceeded through spermatogenesis but without establishing an intralobular gradient of maturation.

Shifting the photoperiod from continuous to natural light in December (LL→NL) resulted in a rapid testicular weight gain during the following 5 months (Fig. 1d), reaching maximum mean GSI values of 7.4 ± 0.6%, similar to those in the control (NL) group. The gradient of maturation was not established in these testes, and the developmental process was

compressed in time compared to control conditions (Fig. 1d). For instance, the period of spermatogonial proliferation was one month shorter than under NL conditions; meiosis and spermiogenesis started one month – instead of two – after the initiation of spermatogonial proliferation; and spermiation started in March and was completed in May (Fig. 1d), i.e. took one month less than in NL controls. Running milt was observed mainly in May and June. At the same time, phagocytosis of spermatozoa by Sertoli cells had commenced, although the highest phagocytic activity was observed in July. The LL→NL group showed a very short – if any – post-spawning resting period, and the second reproductive cycle started as early as August (Fig. 1d). Similar to the developmental sequence of the NL control group, meiosis and spermiogenesis occurred in October and spermiation started in November. In this second season, the maturational gradient in the testicular lobes was present.

Spermatogonial Generations

Spermatogonia in Atlantic cod under NL conditions divided 11 times prior to differentiating into spermatocytes [11]. Pachytene spermatocyte cysts in testis from LL males contained significantly ($p < 0.01$) less cells (1463 ± 425 cells; n = 3) than the number found under NL conditions (1942 ± 360; n = 5; [11]). However, an average in excess of 1400 spermatocytes per cyst is still compatible with passing through 11 mitotic cell cycles. Based on the number of spermatocytes, we calculate that the spermatogenic efficiency up until meiosis under NL conditions was very high (98%) but was reduced to 71% under LL conditions.

Apoptosis

TUNEL-positive germ cells were very rare in the testis of all control (NL) males and, when present, were mainly found among the last spermatogonial generations (Fig. 3a).

In testis of LL-exposed fish, two patterns were found. In fish showing a quiescent testis (55 of 77 males), very little apoptotic germ cells were found (not shown). In LL fish showing spermatogenic activity (27 of 77 males), apoptotic germ cells were much more frequent (Fig. 3b), even though less cysts had been recruited into development. This high germ cell loss was observed in all months and apoptotic cells were randomly distributed in the lobes (supplemental Fig. 2a-f). Most apoptotic cells were late spermatogonia.

The rapid testicular growth and active spermatogenesis triggered by the LL→NL transfer also presented a clearly elevated incidence of spermatogonial apoptosis, but only during the initial two months (January and February 2005) following the shift in photoperiod (supplemental Fig. 2 g-i). When the LL→NL males started the second reproductive cycle in August 2005, spermatogonial proliferation surprisingly again started with an elevated

incidence of apoptosis in August and September (Fig. 3c; supplemental Fig. 2 j and k) while samples from October and November (Fig. 3d; supplemental Fig. 2 l) showed the low level of apoptosis also observed in the control group (Fig. 3a).

Fish moved from NL to LL behaved from May 2005 onwards in a manner similar to the group exposed to LL continuously, i.e. a high incidence of apoptosis was observed in those fish that showed spermatogenic activity in a limited number of cysts.

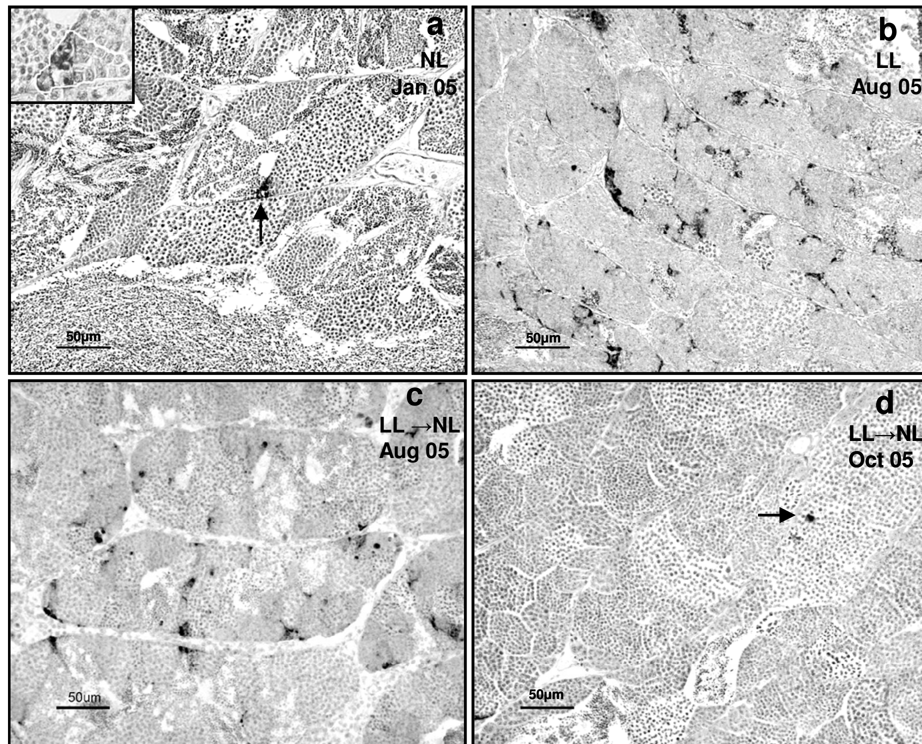

Figure 3: TUNEL labeling of cod testis sections. **a**) NL testis from January 2005 showing a low incidence of apoptosis (arrow); inset shows a higher magnification of a cyst with spermatogonia in apoptosis; **b**) LL testis from August 2005 showing a high incidence of apoptosis; **c**) LL→NL testis from August 2005 showing a high incidence of apoptosis at the beginning of the second reproductive cycle (8 months after the change of photoperiod); **d**) LL→NL testis two months later, in October 2005, when plasma androgen levels had increased and the incidence of apoptosis had returned to the low level typical of NL-exposed fish.

Cod AR *in situ* hybridization

The mRNA for cod androgen receptor (*ar*) was detected in the cytoplasm of Sertoli cells. No labeling was seen when sections were incubated with sense cRNA (Fig. 4b). Not all Sertoli cells showed the same level of *ar* expression, which varied with the stage of spermatogenesis (Fig. 4a). An intense staining was observed in Sertoli cells contacting proliferating spermatogonial clones (Fig. 4c), identified by the size of the germ cells, and by the cytoplasmic

extensions of Sertoli cells between germ cells (see Fig. 4d for comparison). Sertoli cells associated with larger cysts containing spermatocytes showed a progressively weaker labeling, while no label was detected in the large spermatid cysts containing numerous, small germ cells.

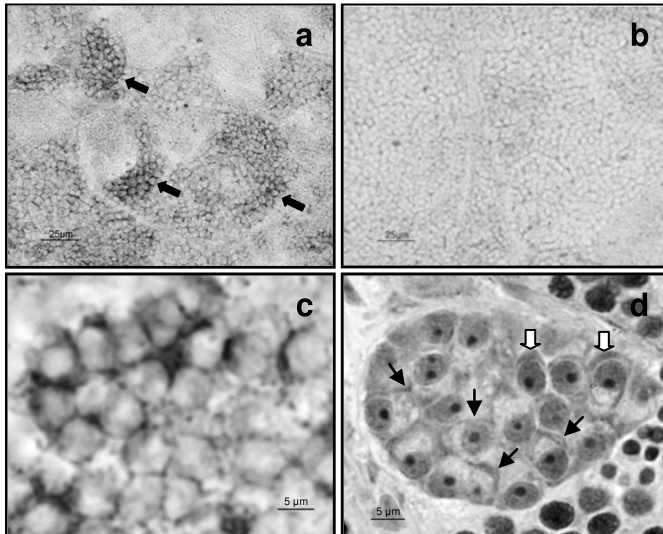

Figure 4: *In situ* hybridization of cod *ar* mRNA in NL testis in October during the rapid growth phase. **a**) antisense cRNA probe showing that the labeling intensity differed between spermatogenic cysts containing germ cells in different stages of development, black arrows indicating spermatogonial cysts; **b**) sense cRNA probe demonstrating the absence of non-specific staining; **c**) antisense cRNA probe in higher magnification revealing the signal in Sertoli cell cytoplasm of spermatogonial cyst; **d**) spermatogonial cyst in a 3 μm plastic-embedded cod testis section, showing

Circulating Androgen Levels

Changes in testosterone (T) levels occurred earlier than those of 11-ketotestosterone (11-KT), while 11-KT reached higher (~ 50%) maximum plasma levels. In the NL group (Fig. 5), T rose moderately from September (significant in October) through December, when also the first significant increase in 11-KT levels was recorded. The levels of 11-KT showed a further 3-fold increase in January and kept rising – though without statistical significance – to a maximum in March, when most males presented running milt. The T levels increased steeply from December to a maximum in January, decreased slightly but significantly in February/March, and fell precipitously to low levels together with 11-KT in April. Both androgens reached minimum values in June. A re-increase at low levels (< 2 ng/ml) started in July, became more prominent as regards T in October and November, when testis growth and spermatogenesis resumed at the beginning of the second reproductive cycle (spermatogonial proliferation).

In LL-treated animals both 11-KT and T plasma levels varied at low concentrations. Maximum levels were recorded in April (Fig. 5) but a specific pattern was not observed.

In the NL→LL group, T levels peaked in January, similar to the control group. However 11-KT values also peaked in January and were significantly higher (1.7 times) than in NL control animals (Fig. 5; Table 1), but decreased clearly already in February, and decreased to

low values already in March (Fig. 5), as opposed to June in the NL group. For the remainder of the study, T and 11-KT plasma levels stayed low in the NL→LL group.

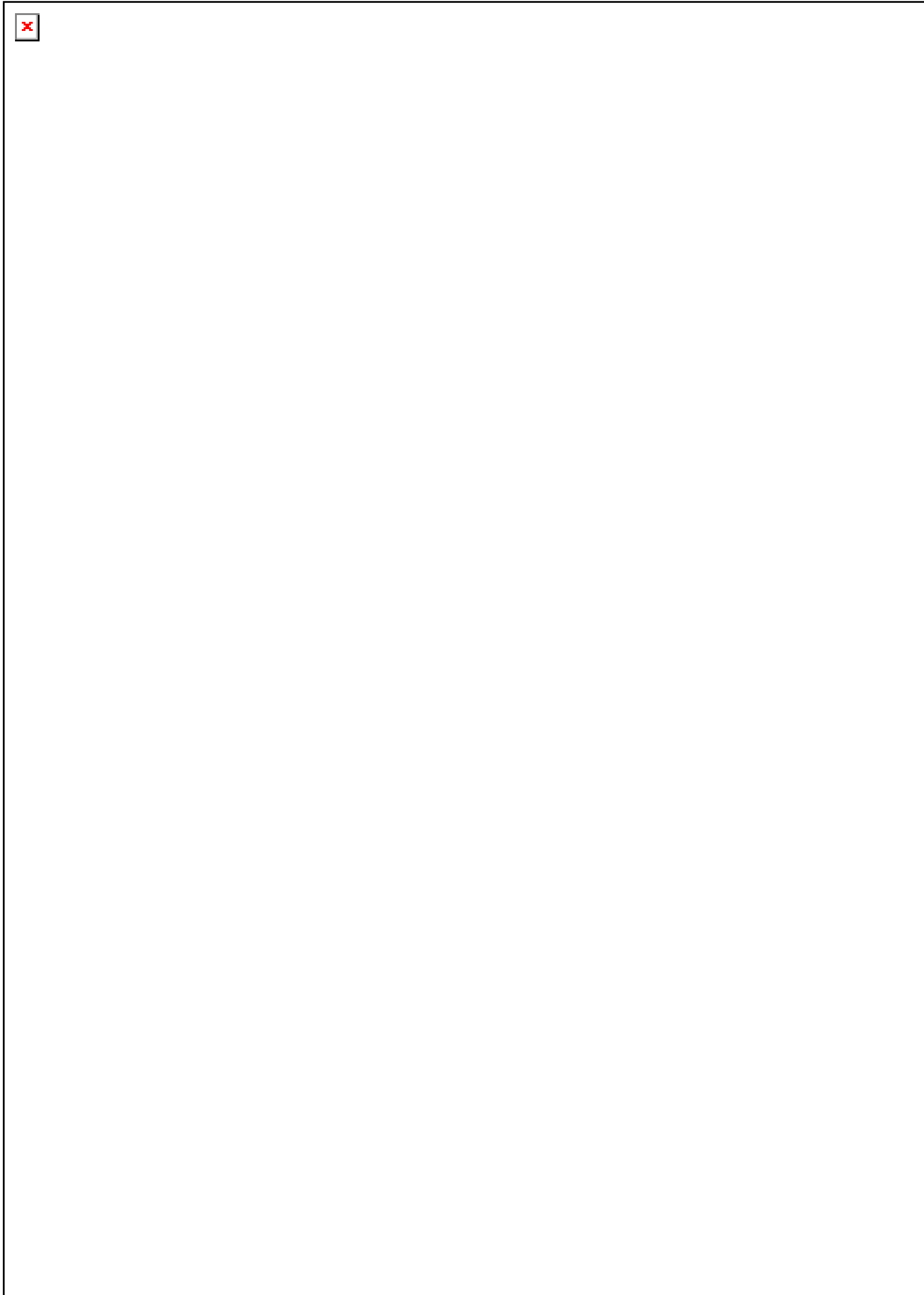

Figure 5: Monthly values of plasma androgen levels (11-ketotestosterone - **11-KT** and testosterone - **T**) and gonadosomatic index (**GSI**) in male Atlantic cod during a 17 mo period. Measurements are from July 2004 to November 2005 (n = 10 - 19). Mean GSI values are indicated on the right ordinate (%) by a dashed line; the steroid values, represented by a continuous line, are shown on the left ordinate (ng/ml; mean ± SEM). The arrows mark the time (December 21, 2004) of the photoperiod change in the NL→LL and LL→NL groups. Means labeled with different letters differ significantly ($P < 0.05$) between the months in the same group; **NL**: normal light; **LL**: constant light. Table 1 gives the results of the statistical comparison of androgen levels.

In the LL→NL group, both 11-KT and T plasma levels started to increase significantly in March (Fig. 5). Peak levels were reached in May and then fell precipitously to low levels in June. Towards the start of the second reproductive season mean plasma androgen levels showed the same pattern as found in the NL control group (Table 1).

Since the incidence of apoptosis among late spermatogonia was high in August and September 2005 in the LL→NL group, but was low after plasma androgen levels rose in October 2005, we examined a possible relation between spermatogonial apoptosis and androgen levels. To this end, we compared plasma androgen levels in individuals showing spermatogenic activity and a low or a high incidence of germ cell apoptosis. As shown in Fig. 6, higher levels of T and 11-KT were found in fish with a low incidence of apoptosis during full spermatogenesis. Statistical significance was reached for T also in animals shortly after the start of spermatogonial proliferation but before meiotic cells were present in the testis.

Table 1 Summary of the statistical comparison of monthly mean plasma androgen levels between the light treatment groups.

	2004						**2005**										
	J	**A**	**S**	**O**	**N**	**D**	**J**	**F**	**M**	**A**	**M**	**J**	**J**	**A**	**S**	**O**	**N**
11-KT																	
NL	a		a			a	a	a	a	a,b	a	a	a				
NL→LL							c	c	b	a	a,b	b	a,b				
LL	b		b			b	b	b	b	a,b	b	b	b				
LL→NL							b	b	b	b	c	a,c	c				
T																	
NL			a	a	a	a	a	a	a	a	a	a				a,b	a
NL→LL							a	b	b	a	a	b				c	b
LL			b	b	b	b	b	b,c	b	a,b	a	b				a,c	b
LL→NL							b	c	c	b	b	b				b	a

Data from July 2004 to November 2005; columns labeled with the same letter do not differ significantly ($P > 0.05$).

DISCUSSION

A possible way to avoid early male maturation in farmed fish is the use of artificial light, a major external cue entraining rhythms of reproduction in fish [15, 35]. In Atlantic cod, photoperiod manipulation modulates the incidence of sexual maturation, delaying it for few months in sea cages, arresting it in indoor tanks or even advancing spawning in compressed

photoperiod [3-5, 18-20, 36]. However, the present study is the first detailed analysis of the effects of photoperiod manipulation on spermatogenesis, including spermatogonial proliferation, the number of mitotic cell cycles, and germ cell apoptosis in fish. An original aspect in the current study on Atlantic cod is the individual correlation of spermatogenic parameters and plasma androgen levels in a large number of animals.

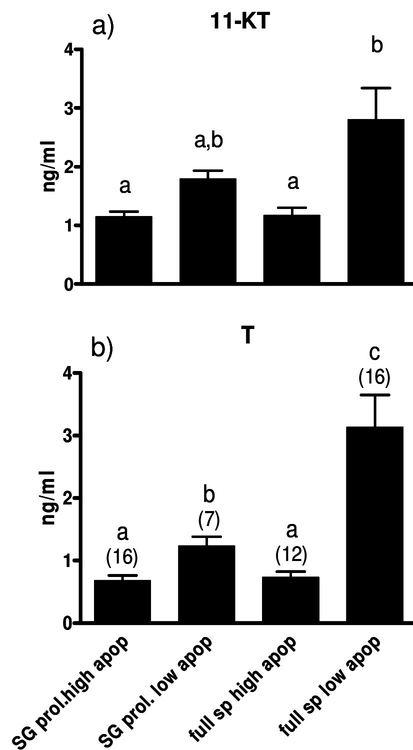

Figure 6: **a**) 11-ketotestosterone (**11-KT**) and **b**) testosterone (**T**) plasma levels (ng/ml; mean) in male Atlantic cod sorted according to the stage of spermatogenesis (spermatogonial proliferation, **SG prol;** full spermatogenesis, **full sp**) and to the incidence of apoptosis. The numbers of samples per group are indicated between brackets. For statistical analysis, the values were log transformed; columns labeled with the same letter do not differ significantly ($P > 0.05$).

Under natural light (NL) conditions, the pubertal maturation of cod testis starts in August with an active proliferation phase of spermatogonia and Sertoli cells, leading to an increase of the number of spermatogenic cysts and germ cell number within the cysts [11]. Meiosis and spermiogenesis start in October and cysts open to release sperm in the tubular lumen from November to February, in preparation for the spawning season from February to April. During this 7 mo period (August to February), spermatogenesis occurs in each individual lobe in a marked gradient of development with the most advanced germ cells in the vicinity of the collecting duct and undifferentiated spermatogonia in the periphery of the lobe, where new cysts are produced, realizing large increases of GSI values by appositional growth of the lobes [11].

Animals exposed to constant light (LL) presented much lower GSI values throughout the study period, and showed low androgen plasma levels. The small changes in average GSI values observed from February 2005 onwards reflected the presence of two response modes to

constant light. Most males (65%) showed early spermatogonia as the only germ cell type in the lobes (quiescent testes) and there was little change in GSI values over time in these individuals. Previous studies showed that exposure of different fish species to constant light similarly delayed or reduced the incidence of male maturation in sea bass (*Dicentrarchus labrax*) [16], turbot (*Scophthalmus maximus*) [37], Atlantic salmon (*Salmo salar*) [38, 39], Senegalese sole (*Solea senegalensis*) [17] and Atlantic cod [4, 18, 20]; the latter study reported reduced androgen plasma levels as well. In sea bass an LL-mediated suppression of androgen plasma levels was associated with reduced pituitary gonadotropin subunits mRNA levels [40].

The other response mode to LL was represented by 27 out of 77 males, where only a limited number of spermatogenic cysts developed but without establishing a gradient of maturation. The two response modes were not related to differences in length, weight or condition factor (relation between body weight and length; data not shown). Instead, the different responses to LL (complete vs. partial inhibition of spermatogenesis) may reflect differences in the individuals' responsiveness of the brain-pituitary-gonad system to the LL challenge. Moreover, the fact that some cysts completed spermatogenesis while most others remained quiescent suggests that each cyst is an independent functional unit that does, or does not, embark on spermatogenesis in response to a given endocrine signaling. The size (in terms of germ cell number) of developing cysts was smaller in LL-exposed than in control fish, but the recruited cysts completed spermatogenesis and reached the spermiation stage. The possibility that under LL conditions less mitotic cell cycles were completed before entering meiosis could be excluded as explanation for the reduced cyst size. Instead, the reduced size of the cysts could be attributed to the high incidence of germ cell apoptosis, in particular among spermatogonia. We therefore conclude that in cod the probably genetically fixed [41] number of mitotic cell cycles is not altered by photoperiod manipulation. Germ cell apoptosis, on the other hand, can be triggered by a wide variety of regulatory stimuli, such as changes in photoperiod, as it is well-known in testis of seasonally reproducing mammals [42, 43]. This is the first time that germ cell apoptosis is described as a consequence of photoperiod manipulation in a teleost species.

The impact of the LL treatment was also demonstrated by the rapid testicular development that took place after the change from the inhibitory LL to the permissive or stimulatory NL conditions. The GSI attained values as high as in NL controls, but peaked in May instead of March, reflecting the delayed start of spermatogenesis. Also Hansen et al. [18] and Davie et al. [20] observed a 3 months delay in spawning activity when cod were transferred from LL to NL during winter. We found in the present study that testis development in the LL→NL group was compressed into 5 months as opposed to 7 months in the control group.

This compressed spermatogenesis took place without establishing the gradient of maturation, and with a high incidence of germ cell apoptosis during the first two months (January and February 2005), reminiscent of the situation in the LL-exposed males that showed some spermatogenic activity, while little apoptosis was observed in animals with rising (March and April) or peaking (May) androgen levels. This increase of plasma androgen levels in the third month after the photoperiod change shows that the immediate response (spermatogenic activity) is temporally dissociated from steroid release. The initially low steroid levels (Jan – Feb 2005) may be responsible for the high incidence of apoptosis during the same period, considering that the incidence of apoptosis decreased when androgen levels started to rise in May 2005. When fish in the LL→NL group embarked on the second reproductive cycle in August 2005, an elevated incidence of spermatogonial apoptosis was again observed in August and September, in contrast to the NL group. Interestingly, the plasma T levels stayed low in the LL→NL group until the onset of meiosis and spermiogenesis but started to rise in the NL group associated with the resumption of spermatogonial proliferation (August/September 2005). In a direct comparison, plasma androgen levels were not different between NL and LL→NL fish in these two months. However, when increasing the sample size by comparing androgen levels in fish from all treatment groups showing a low versus a high incidence of apoptosis, statistically significant differences were found for both androgens and for T also for the period when spermatogonioal proliferation started (Fig. 6). This supports the notion of an androgen-mediated protection against apoptosis during the period of spermatogonial proliferation.

Androgen effects on germ cells are not direct effects but are mediated via the *ar* expressed by Sertoli cells [e.g. 21]. The cod *ar* mRNA expression in Sertoli cells is in line with this notion. Also in adult zebrafish testis, we have recently found *ar* mRNA in Sertoli but not in germ cells [44]. Identification of the cod *ar* mRNA staining pattern as Sertoli cell staining is supported by the very similar staining pattern observed for another Sertoli cell product, the Fsh receptor, in Japanese eel testis [24]. In cod, *ar* mRNA was mainly found in Sertoli cells contacting clones of proliferating spermatogonia. Taken together, it seems that in cod, as in mammals [45], androgens – via Sertoli cells exert protective functions by preventing germ cell apoptosis.

In Japanese eel, 11-KT treatment stimulated spermatogonial proliferation [27]. In Atlantic cod, however, 11-KT plasma levels in the control NL group did not increase significantly from July until December/January (Fig. 5), i.e. four months after the start of spermatogonial proliferation. Plasma T levels, on the other hand, increased already from September in the NL group (see Fig. 5), although the concentrations of T and 11-KT were rather similar from September – November (2-2.5 ng/ml). Preliminary results from ongoing

pharmacological studies indicate that the cod Ar responds to T at ~1 nM concentrations but requires ~10 nM 11-KT. We therefore assume that the Ar expressed in Sertoli cells contacting proliferating spermatogonia may respond mainly to T.

In rainbow trout, Fsh but not Lh plasma levels increased significantly at the beginning of spermatogonial proliferation [46], and the first increase in GSI levels and number of spermatogonia were associated with elevated plasma androgen levels [47]. Since also Fsh is a potent steroidogenic hormone in fish [22, 23], it is possible that Fsh-dependent signaling stimulates spermatogonial proliferation and/or restricts apoptosis, either directly [48] and/or via stimulating steroid production [23, 24, 49].

The spawning-associated peak of 11-KT is compatible with a role for this androgen in regulating reproductive behavior and the development of secondary sexual characters [28, 50]. At the end of the spawning season, 11-KT levels decreased and reached low levels in June/July (NL and LL→NL) or March (NL→LL). Interestingly, the decrease in 11-KT levels preceded (NL→LL, March 2005) or coincided with (NL, April 2005; LL→NL, June 2005) the start of phagocytosis of sperm by Sertoli cells, so that decreasing androgen levels may signal termination of the spawning season.

We hypothesize that photoperiod information is conveyed by central nervous mechanisms to the gonadotropin releasing hormone (Gnrh) neurones controlling the activity of pituitary gonadotroph cells. While the Gnrh-gonadotroph axis is well investigated in fish (reviewed e.g. in [51]), there is little information on mechanisms mediating photoperiod information to the Gnrh system. Although melatonin can serve as a neuroendocrine parameter reflecting the perception of photoperiod cues [52], studies failed to functionally link melatonin and reproduction in lower vertebrates [53], with no evidence of melatonin playing a role in the photoperiodic control of reproduction in teleosts. The LL-mediated inhibition of spermatogenesis and testis growth, as well as the low plasma androgen levels may reflect insufficient gonadotropin signaling. Unfortunately, assays to quantify circulating gonadotropins are not available for Atlantic cod. However, work is ongoing to characterize cDNAs encoding pituitary hormones (*lh*, *fsh*) and their gonadal receptors from Atlantic cod. Quantification of the respective mRNAs may shed more light on the regulatory background of photoperiod-induced modulation of testis development.

In summary, constant light in indoor tanks either silenced testis development completely or severely restricted the number of spermatogenic cysts recruited into spermatogenesis, in association with elevated levels of apoptosis among the spermatogonia that did form. Both effects, i.e. reduced recruitment and increased apoptosis, were associated with reduced androgen levels that, in turn, may reflect insufficient gonadotropic stimulation.

ACKNOWLEDGMENTS

We gratefully acknowledge the skilled assistance of the technical staff at the Austevoll Research Station for running the experiment and helping in sampling. Ms. Svanhild Lohne Gokstad and Ms. Sissel Waage Kalvenes are acknowledged for assistance with steroid assays.

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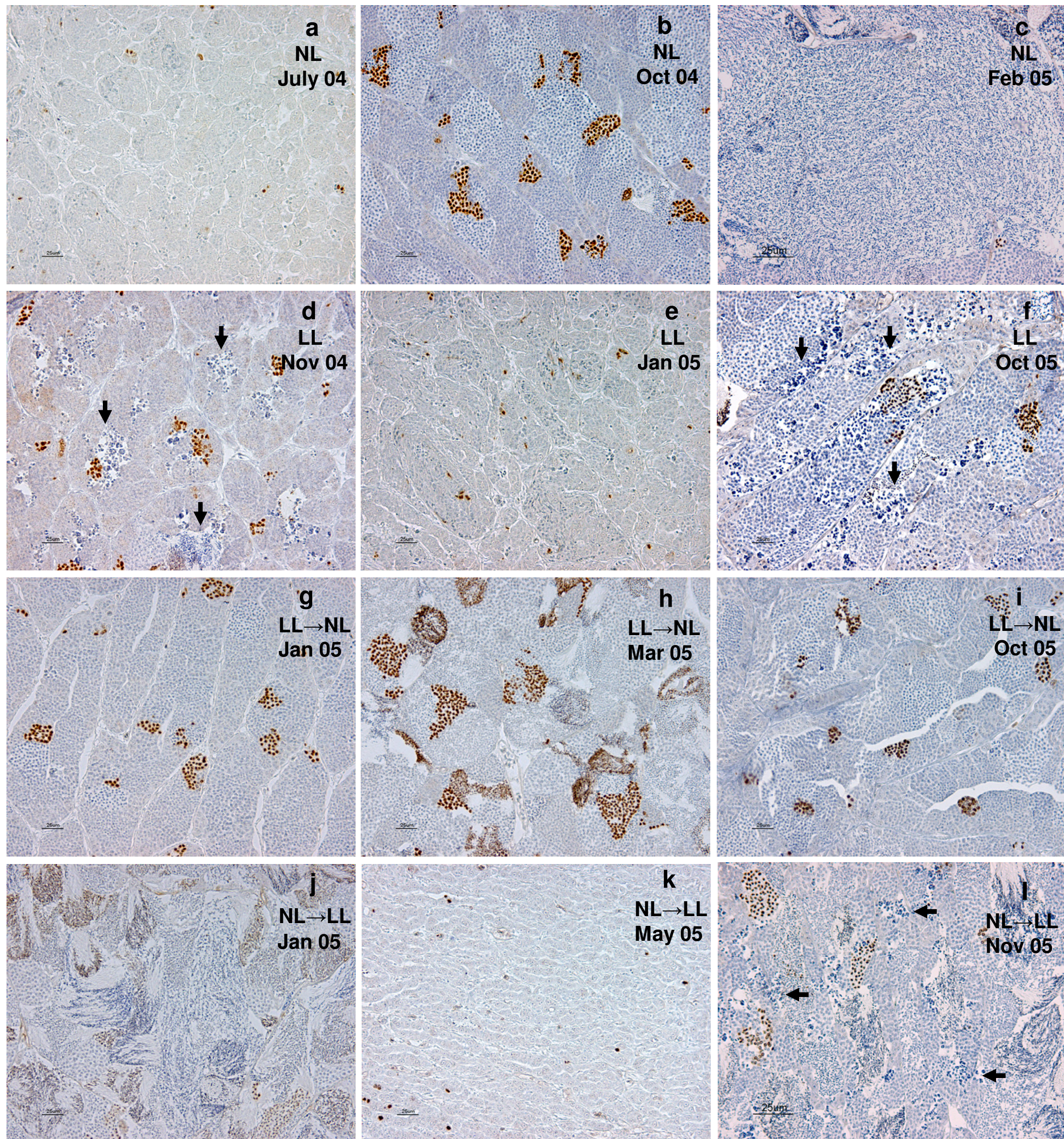
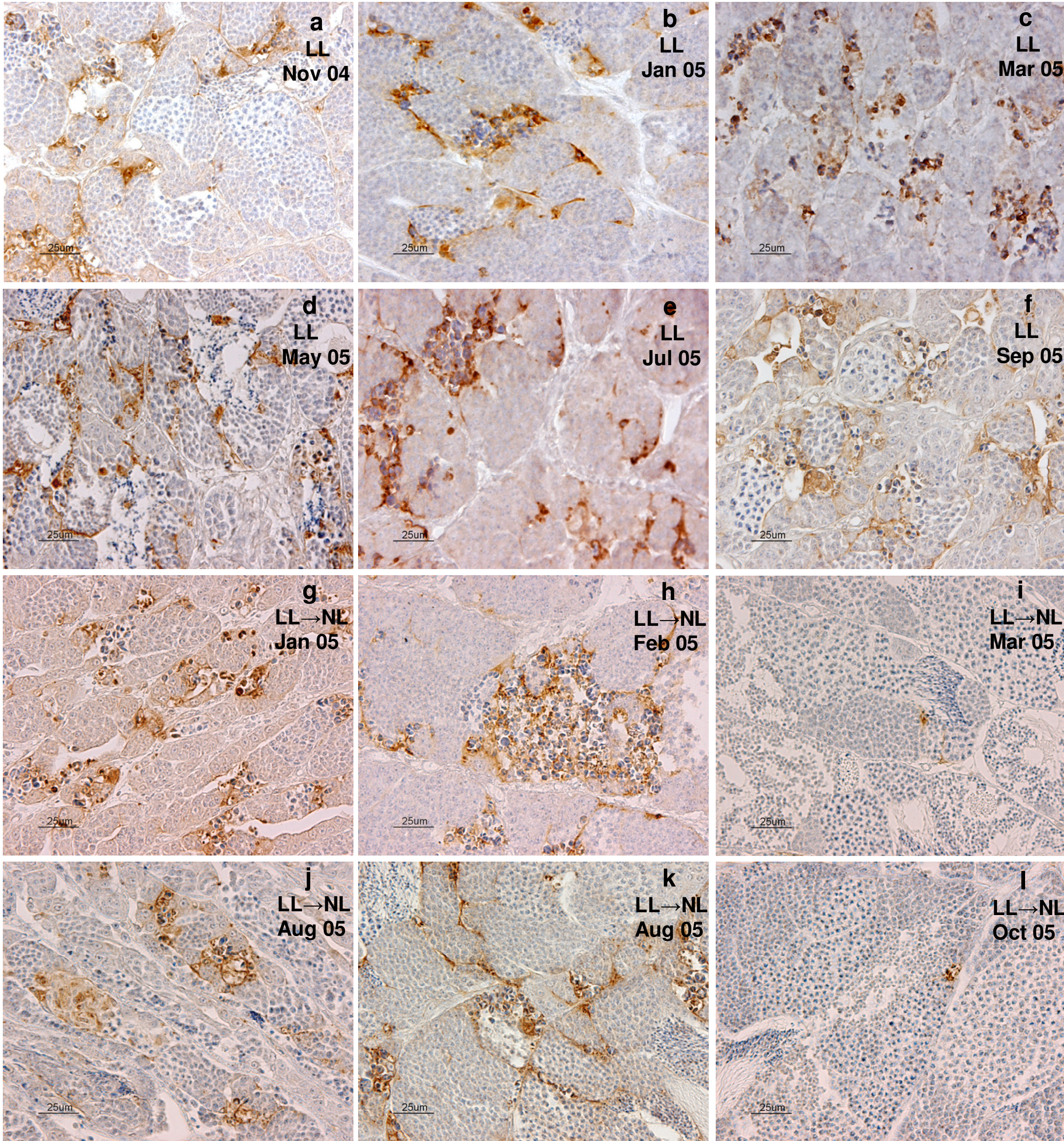
Supplemental Table 1: Number of animals analyzed for proliferation (pH3) or apoptosis (TUNEL) from the different photoperiod treatment group per month.

	2004						2005										
	J	**A**	**S**	**O**	**N**	**D**	**J**	**F**	**M**	**A**	**M**	**J**	**J**	**A**	**S**	**O**	**N**
pH3																	
NL	9	4	4	12	10	8	17	7	8	6	9	5	4	10	6	6	6
LL	6	2	3	8	5	5	11	14	5	3	3	4	5	9	9	11	8
NL→LL							16	11	8	6	7	3	2	3	4	5	5
LL→NL							13	10	9	4	6	4	4	15	14	9	9
TUNEL																	
NL	0	1	3	6	6	5	3	1	1	2	4	2	1	3	6	6	5
LL	0	2	3	7	5	4	9	8	3	3	3	4	4	9	8	11	8
NL→LL							16	11	5	5	6	3	2	3	4	5	5
LL→NL							8	5	5	3	4	4	4	15	14	9	9

Samples from July 2004 to November 2005;
pH3: Phosphorylated Histone H3; NL: normal light; LL: constant light



Supplemental Figure 1: Immunohistochemistry for phosphorylated histone H3 (pH3) in cod testes. NL testes before (**a**), during (**b**) and after (**c**) rapid spermatogonial proliferation; LL testes during spermatogonial proliferation (**d**), quiescence (**e**) or full spermatogenesis (**f**); LL→NL testes after light change at the beginning of spermatogonial proliferation (**g**), full spermatogenesis (**h**) and second cycle (**i**); NL→LL testes after light change (**j**); quiescent (**k**) or full spermatogenesis (**l**). Arrows point to apoptotic germ cells (highly condensed nuclei) in LL and NL→LL groups.



Supplemental Figure 2: TUNEL labeling of cod testes. Testes from every other month from the LL group **(a** to **f**); testes from the LL→NL group during spermatogonial proliferation in the presence of low androgen levels in the first (**g** and **h**) and second (**j** and **k**) cycles, and after the rise of plasma androgen levels(**i** and **l**).

4

Pituitary Gonadotropin and Testicular Gonadotropin Receptor Expression in Atlantic Cod (*Gadus morhua*, L.) During the First Reproductive Season: Effects of Photoperiod Modulation

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ABSTRACT

The control of gonadal functions by the two gonadotropins, follicle-stimulating hormone (Fsh) and luteinizing hormone (Lh) via their cognate receptors is a well known mechanism triggering puberty in vertebrates. Prepubertal male cod were exposed in covered tanks for 17 months to different photoperiods known to affect pubertal testis growth. We then studied pituitary expression of gonadotropins and testicular expression of gonadotropin receptors, and correlated this with pubertal testis development. Increasing expression of *fshb* was noted during the initiation of testis development, while *fshr* expression showed less prominent changes during the annual cycle. At later stages of spermatogenesis, towards and during spawning, *lhb* mRNA and plasma androgen levels increased as did, one month later, also *lhr* mRNA. Cod responded in two different manners to exposure to continuous light. When given in the summer, it suppressed increases in gonadotropin subunit and gonadotropin receptor mRNA levels, while when given in winter it furthered up-regulated *lhb* and *lhr* expressions and androgen plasma levels, finally resulting in a precocious termination of the spawning season.

INTRODUCTION

Teleost fish are typical vertebrates in that pubertal testis maturation and adult reproduction are controlled by the endocrine system. The brain receives signals from internal and external sources, such as endocrine feedback or signals mediating information on the nutritional or health status, and environmental cues (e.g. photoperiod), and integrates the information into regulatory output that eventually leads to changes in gonadotropin-releasing hormone (Gnrh) release towards the pituitary gonadotroph cells. These cells produce follicle-stimulating hormone (Fsh) and luteinizing hormone (Lh), which jointly regulate spermatogenesis and hormone/growth factor production in the testis (McLachlan *et al.* 1996; Themmen and Huhtaniemi 2000; Meng *et al.* 2000). Some of the gonadal hormones, e.g. sex steroids or the growth factor inhibin, are important signalling molecules in feedback loops from the gonad to the brain and/or the pituitary, rendering the brain-pituitary-gonad (BPG) axis into an integrated regulatory system, exerting the master control over puberty and adult reproduction.

In previous chapters, we have dealt with the spermatogenic process during pubertal testis maturation (Chapters 2 and 3), and have analysed androgen plasma levels under different photoperiod regimes (Chapter 3). In Chapter 5, we characterise the androgen receptor and its expression pattern. The present chapter will concentrate on the interaction between the pituitary and testis by investigating the expression of the pituitary gonadotropins and their cognate receptors. It would have been interesting to extend these studies to signalling systems further upstream of the pituitary level, such as Gnrh neurones, or to the input they receive via the Kiss1/Gpr54 system (van Aerle *et al.* 2008; Felip *et al.* 2008). However, these topics were outside the scope of the present thesis and are subject to presently ongoing research.

The main domains of activity of pituitary gonadotropins and their cognate receptors are the regulation of gonadal hormone and germ cell production. Loss-of-function models in mammals revealed valuable information about the role of gonadotropins. Proper function of Fsh/Fshr is necessary for normal Sertoli and germ cell number, testosterone levels, and sperm morphology (Singh *et al.* 1995; Krishnamurthy *et al.* 2000; Wreford *et al.* 2001; Haywood *et al.* 2003) while in the absence of Lh spermatogenesis is arrested at the spermatid stage (Cattanach *et al.* 1977; Zhang *et al.* 2001, 2004). Also in fish, removal of the pituitary gland (Billard 1969a; Liley and Donaldson 1969; Khan *et al.* 1986 and 1987), exposure to conditions that block activation of the brain-pituitary-testis axis (eg. preventing migration into the marine environment in eel: Sébert *et al.* 2008), the timing of gonadotropin receptor expression in relation to natural sex change and the ensuing testis development (Kobayashi *et al.* 2009), or testis tissue culture studies using recombinant gonadotropins (Ohta *et al.* 2007) all provide clear evidence for the dependency of pubertal testis maturation/spermatogenesis on pituitary signalling.

Gonadotropin receptors are structurally well conserved among vertebrates (Ji *et al.* 1998; Vassart *et al.* 2004; Blomenröhr *et al.* 2002), but cellular expression patterns and hormone binding characteristics can differ between mammals and fish. In the former, the Fshr is exclusively expressed by the somatic Sertoli cells in the germinal compartment that control germ cell development, while Lhr is mainly expressed by Leydig cells, which are located in the interstitial compartment of the testis and are responsible for steroidogenesis in the testis. In fish, Lhr is similar to its mammalian counterpart and specifically responds to Lh, while the Fshr responds mainly to Fsh but also to (high levels of) Lh, such as during the spawning season (African catfish, Vischer *et al.* 2003; Channel catfish, Zmora *et al.* 2007; zebrafish, So *et al.* 2005; Japanese eel, Kazeto *et al.* 2008; Pacific salmon, Yan *et al.* 1992; Miwa *et al.* 1994). Moreover, next to Sertoli cells, in some species such as Japanese eel and African catfish, also Leydig cells express the Fshr protein (discussed in García-López *et al.* 2009). Hence, the clear separation of

tasks between the two gonadotropins in mammals seems less strict in fish, at least in certain species.

In fish species living at moderate or high latitudes, pubertal development and adult reproduction typically are seasonal events associated with impressive changes in testicular mass, cellular composition and sex steroid output (Bromage *et al.* 1993; Weltzien *et al.* 2002; Almeida *et al.* 2009). Data on puberty-associated changes in pituitary gonadotropin expression are available from a number of fish species (e.g. trout – Prat *et al.* 1996; sea bass – Rodriguez *et al.* 2000, Mateos *et al.* 2003; Atlantic halibut – Weltzien *et al.* 2003; Japanese flounder – Kajimura *et al.* 2001; fathead minnow – Filby *et al.* 2008), while information on changes in the plasma levels of both Fsh and Lh are restricted to salmonids (Gomez *et al.* 1999; Santos *et al.* 2001; Prat *et al.* 1996; Larsen and Swanson 1997; Campbell *et al.* 2003). Previous work in different species also has shown that pituitary gonadotropin subunit expression, gonad growth, and/or plasma steroid levels are very sensitive to photoperiod manipulation (e.g. sea bass, Rodriguez *et al.* 2005; Atlantic cod, Taranger *et al.* 2006; Norberg *et al.* 2004; Atlantic salmon, Schulz *et al.* 2006). Information on the maturation-associated changes in testicular gonadotropin receptor expression is beginning to emerge only, is restricted to two closely related species of salmonids, and shows a certain variation between studies (Kusakabe *et al.* 2006; Sambroni *et al.* 2007; Maugars and Schmitz 2008). However, no information is available on the effects of experimental conditions, such as different photoperiod regimes known to strongly modulate pubertal development in males, on the expression of pituitary gonadotropins or testicular gonadotropin receptors.

Puberty in male Atlantic cod is particularly sensitive to photoperiod manipulation and it seems reasonable to assume that expression of pituitary gonadotropin subunits and/or their testicular cognate receptors is involved in mediating photoperiod-modulated testis maturation. The recent cloning of the gonadotropin subunits and their cognate receptor cDNAs as well as the development of specific quantification systems for the respective mRNAs (Mittelholzer *et al.* 2009a,b) were instrumental to carry out the present study on photoperiod-manipulated puberty. The choice of the species is moreover influenced by the fact that wild stocks of Atlantic cod are declining. As a result, cod aquaculture is of increasing interest in countries around the North Atlantic. However, under the favourable growth conditions in aquaculture, male cod often become sexually mature before the fish reach market size, and sexual maturation has a negative effect on the growth performance and represents a risk of genetic "contamination" of remaining wild stocks (Jørstad *et al.* 2008). Consequently, early puberty is a significant bottle-neck regarding different sustainability aspects of cod aquaculture.

The overall objectives of our study therefore are to further our knowledge on the

endocrine regulation of pubertal testis maturation in fish, by analysing the expression pattern of cod Fsh and Lh transcripts in the pituitary and of their cognate receptors in the testis under normal light conditions, and under light conditions that suppress or accelerate testis maturation.

MATERIAL AND METHODS

Animals, Light Regimes and Samples

Prepubertal Atlantic cod (Norwegian Coastal Cod) were reared in 3 m diameter, 1 m water height (7 m^3) seawater outdoor tanks at the Institute of Marine Research, Austevoll Research Station, Norway (60^0N). The larvae hatched March 2003 and were first fed on natural zooplankton in a semi-enclosed seawater pond before transfer to the experimental tanks, where they were fed a commercial dry pellet diet (DanEx 15-62, Danafeed, Horsens, Denmark) *ad lib*. The tanks were supplied with seawater pumped from 168 m depth and the water temperature ranged from 7.4 to 9.4^0C (mean ± SD = 8.1 ± 0.3^0C) during the experimental period. All fish were treated and euthanized according to Norwegian National Legislation for Laboratory Animals (1996).

From July to December 2004 the animals were divided into two experimental groups, each housed in 6 replicate tanks. One group was reared under natural light (NL) (tanks covered with 70% light reducing shading nets), the other group was exposed to continuous light (LL) in light proof tanks. The light was supplied by 70W metal halide lamps (Phillips MHW-TD 70W light bulbs), giving an integrated irradiance of 5.5×10^{-4} W s^{-1} cm^{-2} just below the water surface. Starting December 21, three tanks of each group were exposed to the other light condition until November 2005, resulting in four experimental groups: 1. NL throughout; 2. LL throughout; 3. six months of NL followed by eleven months of LL (NL→LL); and 4. six months of LL followed by eleven months of NL (LL→NL; Figure 1).

Every month, 30 animals were sampled randomly from all groups, among which 10-19 were males; however, the sex ratio was close to 50% integrated over all samplings. The entire pituitary and a sample of testis tissue were dissected, wrapped in aluminium foil and immediately snap-frozen by immersion in liquid nitrogen, before storage at -80^0C for later RNA extraction and cDNA synthesis. Histological and immunocytochemical analysis of testis development and androgen quantification in blood samples of the same animals has been reported previously (Almeida *et al.* 2009). There was no statistically significant difference in gonado-somatic index between animals from the six or three replicate tanks from the same light

treatment, so that all data were pooled according to the photoperiod regime. The mortality was low with an overall loss of 9% over the 17 months period.

RNA extraction and Reverse Transcription

Pituitaries from 6-5 males per month and group and testis tissue from 3-10 males per month and group were weighed and quickly transferred to cold Tri Reagent (Sigma-Aldrich, Oslo, Norway). Total RNA was prepared from individual pituitary/testis samples by the acid phenol-guanidine thiocyanate method after tissue homogenization in the FastPrep tube containing Lysing Matrix D ceramic beads; and quantified by spectrophotometry after overnight precipitation with isopropanol. DNase treatment of 10 µg of total RNA (TurboDNA-free, Ambion/Applied Biosystems, Oslo, Norway) was applied to testis RNA samples before reverse-transcription procedure. Random-primed cDNA was synthesized from 500 ng of DNase-treated RNA using the Reverse Transcriptase Core Kit (RT-RTCK-05, Eurogentec, Belgium).

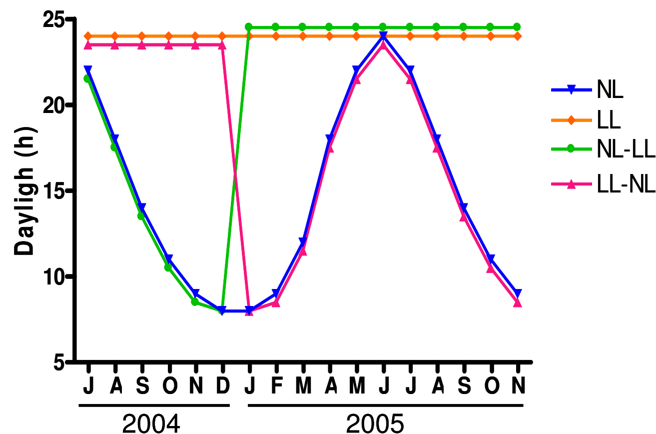


Figure 1. Schematic representation of the four light regimes. **NL**: normal light; **LL**: constant light. Data from July 2004 to November 2005.

Quantitative real-time PCR

Quantitative real-time PCR assays for the gonadotropin subunits (*fshb* and *lhb*) and their cognate receptors were designed as described in Mittelholzer et al. (2009 a and b, respectively).

For the *fshb* and *lhb* assays, exon-exon boundaries were spanned in the mRNA sequence to prevent genomic DNA amplification. The primers used to quantify *fshr* and *lhr* mRNA expressions were directed to sequences located in the transmembrane domain in order to

amplify all alternative splicing variants containing a membrane anchoring domain. All sets of primers were subjected to an initial testing by running them in a conventional PCR on both cDNA and genomic DNA.

The PCR reaction solution contained 900 nM of primers, 200 nM of TaqMan probe, 1X AmpliTaq Gold TaqMan Mix (Ambion/Applied Biosystems) and 2 µl template (diluted 1:10 in nuclease-free water) in a 25 µl total volume. The PCR profile was as follows: 50^0C for 2 min, 95^0C for 10 min, 40 cycles at 95^0C for 15s and 60^0C for 1 min, followed by 72^0C for 10 min. For each sample, two PCR reactions were performed and the results were averaged. Data were analyzed by Sequence Detector version 1.6.3 (Ambion/Applied Biosystems). *fshb, lhb, fshr* and *lhr* gene expressions were calculated with the ΔΔCt method, as described in detail previously (Bogerd *et al.* 2001). The results were expressed as relative quantities of gene expression (rel Q), normalized to elongation factor 1-α (*ef1a*) as reference gene. Controls without cDNA were also run in duplicate for each qPCR reaction plate.

Statistical Analysis

The differences in gene expressions (*fshb*, *lhb*, *fshr* and *lhr*) between different months in the same group (NL, LL, NL→LL or LL→NL) were assessed by analysis of variance (ANOVA) on log-transformed relative quantity values, followed by Tukey HSD post-hoc test (unequal n). For comparing gene expression in different treatments in the same month, Student *t* test was used from July to December 2004 (comparing NL to LL) and ANOVA with Tukey HSD test from January to November 2005 (comparing all groups).

RESULTS

Pituitary Gonadotropin Expressions

The mRNA levels of the two gonadotropin β-subunits showed significant changes during testis maturation as well as in response to the different light regimes (Fig. 2).

Under normal light (NL) conditions, pituitary *fshb* mRNA expression increased ~5-fold from July to October 2004 and remained at high levels until December. A partial decrease was recorded for the period from January to March 2005, before basal levels were reached in April and maintained until August. The *fshb* mRNA levels re-increased starting in September (Fig. 2a), when an increase in spermatogonial proliferation heralded the start of the second cycle.

In fish submitted to constant light (LL group), we did not find major variations in the pituitary expression of *fshb* during the entire experiment, although statistical analysis showed

that August 2004 differed significantly from April 2005. The monthly mean values ranged only from 0.9 to 2.6. A summary of the statistical analysis of the differences between the photoperiod treatment groups is given in Supplemental Table. The main difference between the NL and LL groups were the absence of *fshb* up-regulation during late summer/fall in the LL group, and a stronger down regulation before the start of the second cycle in the NL group.

After the change in photoperiod from NL→LL in December, *fshb* mRNA amounts initially (in January) were high. However, down-regulation to basal levels occurred already in February, i.e. two months earlier than in the control (NL) group. After the March nadir, *fshb* expression increased slightly to the levels also found in the LL group, and showed the same pattern as this group also for the remainder of the experiment (Fig. 2a).

In the LL→NL group, *fshb* transcripts increased slightly after the photoperiod shift in January and significantly in February (Fig. 2a). Expression was maintained at elevated levels until May, i.e. during the period of full spermatogenic activity (please refer to the gonadal stage of development of the LL→NL group along the x-axis in Fig 3b). Different from the NL group, where first a partial down-regulation of expression was observed from January to March, the period of high levels was immediately followed by a period of low levels (June and July) in the LL→NL group. From August onwards, concurrent with the start of spermatogonial proliferation for the second cycle, *fshb* mRNA levels re-increased, reaching statistical significance in October and November 2005 (Fig. 2a).

Different from *fshb*, cod *lhb* pituitary expression did not change in males in the NL group from July through November 2004. However, from December 2004 *lhb* expression increased until reaching maximum values in February 2005 (Fig. 2b), when the spawning period started. Down-regulation of *lhb* mRNA started towards the end of the spawning season in April, declining steadily to minimum values in July 2005. Thereafter, a slight, statistically not significant, rise was observed until the end of the experiment in November 2005.

The *lhb* mRNA expression in the pituitary of fish kept under LL conditions varied at relatively low levels throughout the 17 months of the experiment, without displaying distinct peaks (Fig. 2b), although the first 3 months were lower than the mean value in April 2005. The main difference to the NL group was the absence of an up-regulation of gene expression in the winter in the LL group, and the lower level in the NL group in July 2005.

In fish moved from NL to LL conditions, the *lhb* mRNA levels showed a strong, up-regulatory response in January (Fig. 2b). High levels were maintained in February, when high levels were also observed in the NL control group. Different from the latter, a sharp decrease started already in March, i.e. one month earlier than in the NL group, and continued in April. Thereafter, the relative *lhb* mRNA amounts varied between 2.2 and 4.6, with little difference

between months or to other treatment groups.

In the LL→NL group that showed an out-of-season testis development following the switch to NL conditions at the end of December, we recorded steadily increasing *lhb* mRNA levels from January onwards, reaching statistical significance compared to all other groups in May and June (except NL→LL in June), i.e. shortly and during the start of this group's spawning season (Fig. 2b).

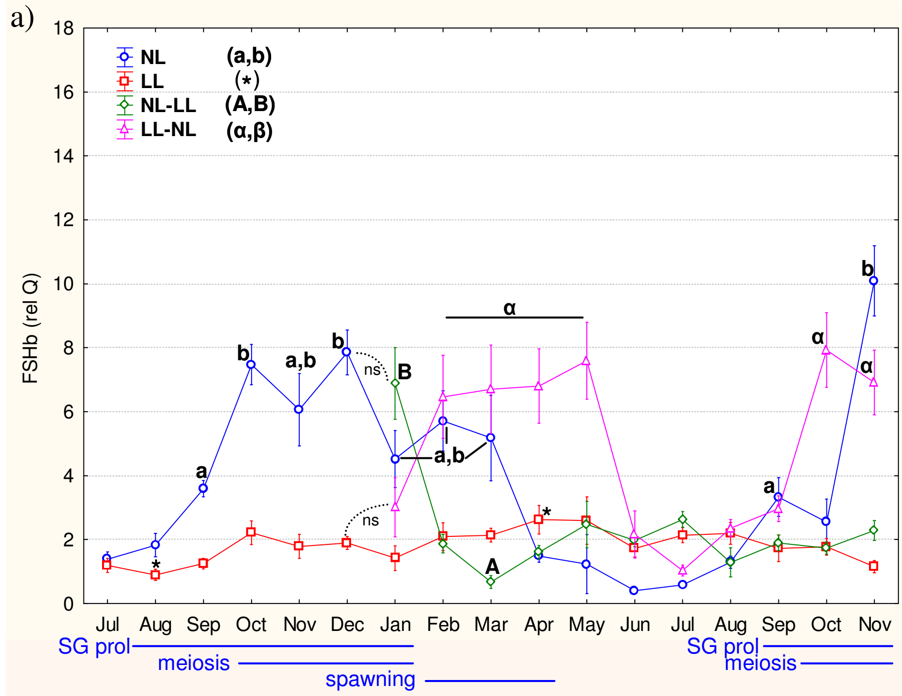
Previous work showed that spermatogenesis in cod can respond in two ways to continuous light applied from the summer solstice onwards, namely either staying quiescent, or showing a quantitatively and qualitatively restricted spermatogenesis (Almeida *et al.* 2009). We therefore wondered if sorting the data from LL males into the two subgroups 'quiescent' (n = 26) and 'spermatogenesis' (n = 24) would reveal significant differences. Statistical analysis showed that *lhb*, but not *fshb*, mRNA levels were higher in males showing spermatogenic activity (Fig. 6), although the levels stayed 3-4 fold lower than the maximum levels found in the NL or LL→NL groups. Moreover, plasma T, but not KT, levels were significantly higher in developing males.

Gonadotropin Receptor Expressions in Cod Testis

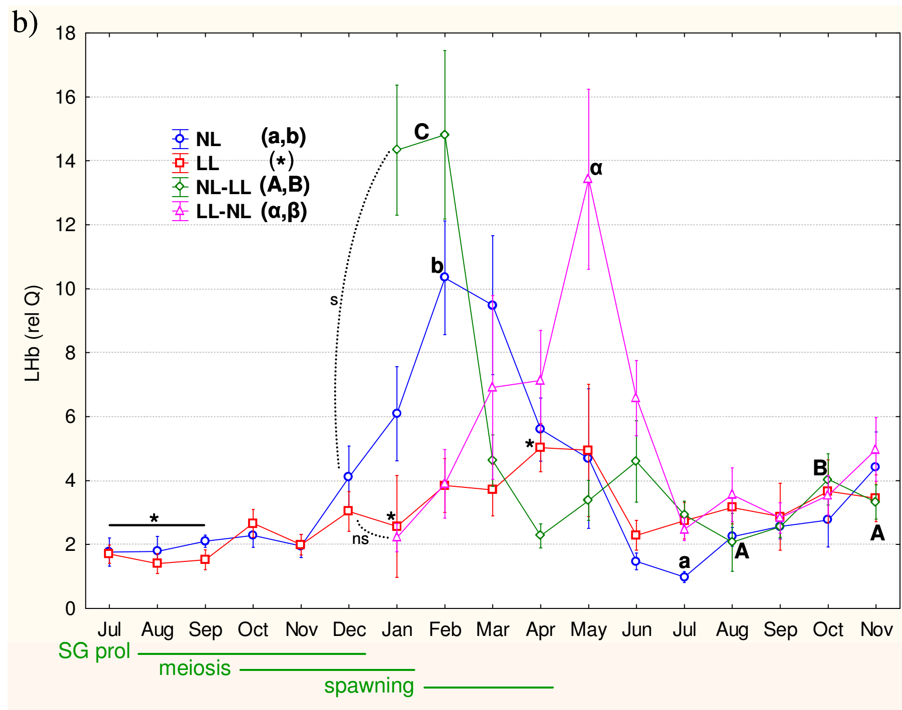
The *fshr* mRNA levels showed little variation during the entire experimental period with regard to both, seasonal changes in the control (NL) group and effects of photoperiod treatment regimes. However, the levels were always clearly detectable, so that *fshr* expression appears to have a considerable constitutive component. The *lhr* mRNA levels, on the other hand, showed distinct peaks in association with testis development and in response to the experimental photoperiod regimes (Fig. 3).

Two remarkable trends were observed among the relatively small changes in *fshr* mRNA levels. First, at the beginning of testis growth, relatively high levels were found that decreased significantly to lower levels in testis during full spermatogenesis. This was observed three times: in the NL group from high levels in July to low levels in September 2004; from high levels again in July 2005 that decreased towards November 2005; in the LL→NL group, in which testis growth was triggered by the shift to NL, from relatively high levels in December 2004 to low levels in March 2005. Second, elevated *fshr* expression were observed during the peak spawning season in both the NL (March 2005) and the LL→NL (June 2005) groups (Fig 3a).

No significant changes in the *fshr* expression were observed in the LL group (Fig. 3a). Comparing the quiescent and spermatogenic subgroups under LL conditions revealed higher *fshr* expression in quiescent testes (Fig. 6).



* Only significant difference in LL group



* Significant difference in the LL group only in April 2005 being higher than July, August and September 2004, and January 2005.

Figure 2. Relative expression of cod pituitary gonadotropins. a) *fshb* mRNA relative quantity (**rel Q**). b) *lhb* mRNA relative quantity. **NL**: normal light; **LL**: constant light. **ns**: not significant change after light shift; **s**: significant change after light shift. **SG prol**: spermatogonial proliferation. Letters and symbols (identified between brackets close to group legends) show differences between months in the same group and for clarity were added from the first significant difference. Histology data refers to NL group in a (in blue) and to NL→LL in b (in green). Data from July 2004 to November 2005.

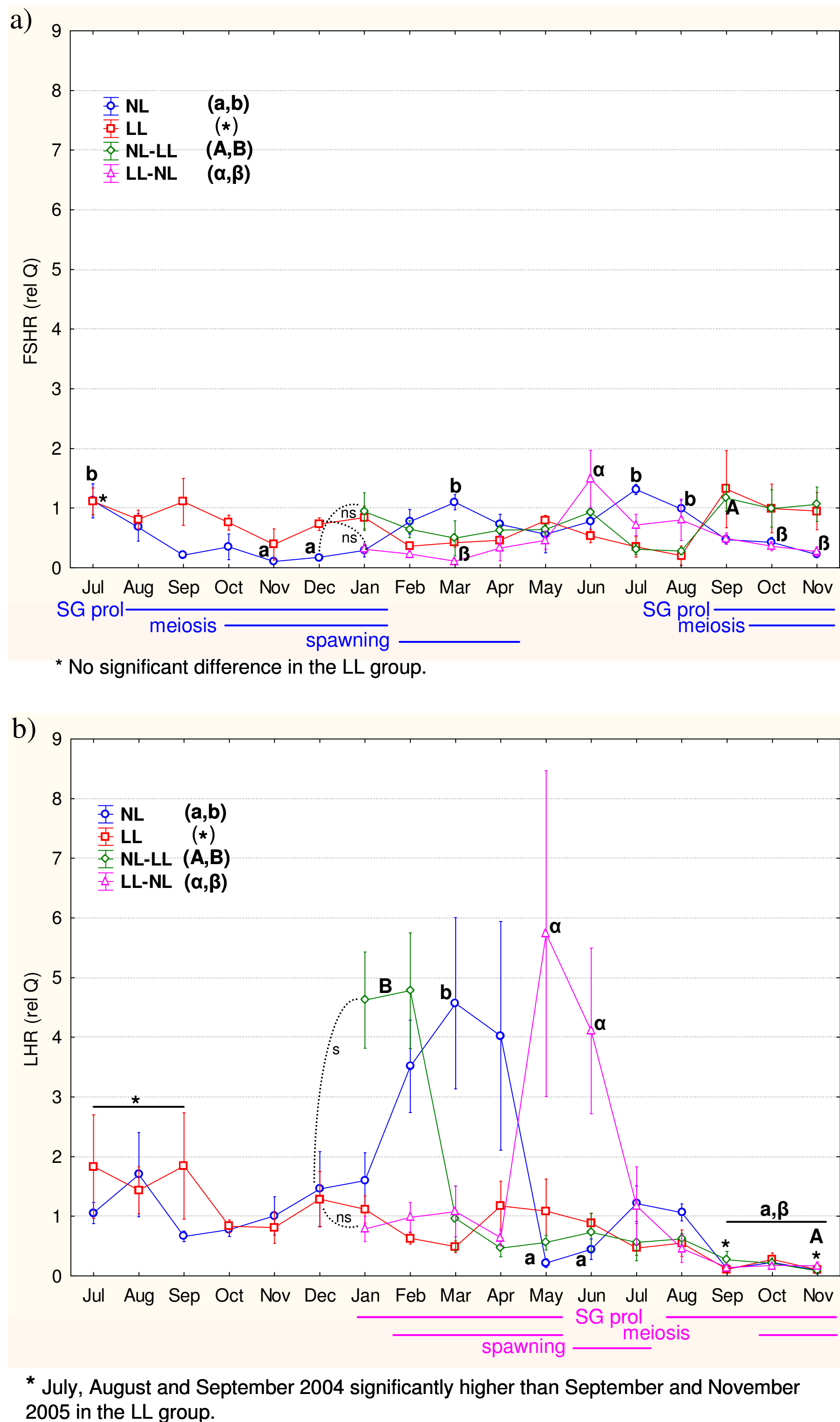


Figure 3. Relative expression of cod testis gonadotropin receptors. a) *fshr* mRNA relative quantity (**rel Q**). b) *lhr* mRNA relative quantity. **NL**: normal light; **LL**: constant light. **ns**: not significant change after light shift; **s**: significant change after light shift. **SG prol**: spermatogonial proliferation. Letters and symbols show differences between months in the same group and for clarity were added from the first significant difference. Histology data refers to NL group in a (in blue) and to LL→NL in b (in pink). Data from July 2004 to November 2005.

In testis samples of the NL→LL fish, *fshr* expression tended to increase after the light change without reaching statistical significance. For the remainder of the experiment, this group behaved very similar to the group subjected to LL conditions throughout. From June 2005, the LL and NL→LL groups were superimposed as regards their *fshr* mRNA levels (Fig. 3a).

The testicular *lhr* expression in cod kept under NL conditions was low during the beginning of pubertal testis development (Fig. 3b). Up-regulation of *lhr* expression became evident in February, reached statistical significance in March, and stayed at high levels in April. A rapid decrease was observed in May. The relatively small increase during the summer 2005, similar to that in the summer before the first reproductive cycle, did not reach statistical significance (Fig 3b).

Fish from the LL group presented low testicular expression of *lhr* mRNA during the entire period studied. Significant differences were apparent only when comparing animals from the first three to the last month (Fig. 3b), and when comparing the quiescent with the spermatogenic LL subgroups (Fig. 6).

In the NL→LL group, the *lhr* mRNA levels were strongly up-regulated in response to the photoperiod change at the end of December. The levels were still high in February but the significant difference to the control group was lost, as the latter had increased as well in February (Fig. 3b). A strong down-regulation was recorded in March, and from April onwards no significant changes were recorded in this group that behaved increasingly similar to the LL group.

The expression of *lhr* in testis from LL→NL fish remained low during the first four months after the photoperiod shift. A sharp peak was quantified in May and down-regulation appeared to start in June already (Fig. 3b). Low levels without significant differences were found until the end of the study in November.

DISCUSSION

The activation of the brain-pituitary-gonad axis triggers puberty, assuring a juvenile to acquire the capacity to reproduce. At the start of puberty, the two gonadotropins secreted by the pituitary, Fsh and Lh, via activation of their cognate receptors in the gonads, regulate events involved in pubertal gonad maturation, steroid synthesis and gamete production. Hence, in the present study, we analyzed the expression of the genes encoding pituitary gonadotropins and their receptors as crucial parameters for pubertal testis maturation in Atlantic cod. Our main findings as regards the Fsh/Fshr signaling system were that *fsh* synthesis is clearly up-regulated

during testis growth, while *fshr* expression showed relatively small changes (compared to *lhr* mRNA) during testis development. A partial down-regulation in *fsh* was observed when plasma androgen levels increased steeply in spawning fish. In spent fish, *fshb* mRNA levels were fully down-regulated and stayed low until the start of the second cycle of testis growth. In the Lh/Lhr signaling system, both ligand and receptor genes were clearly regulated, changed in a similar manner, and moreover in association with plasma androgen levels (up-regulation towards spawning; down-regulation towards the spent condition). Finally, exposure to experimental photoperiods induced significant changes in the expression patterns of all four genes, and alterations in the pituitary *lhb* usually preceded those of its receptor gene.

The decrease in day-length that starts after the summer solstice is perceived via the lateral eyes and/or the pineal organ (Falcón *et al.* 2007). We speculate that the decreasing photoperiod is the environmental signal that stimulated the release of gonadotropin-releasing hormone (Gnrh) by hypothalamic neurosecretory neurons that send their axons directly into the pituitary, ending in the vicinity of the gonadotrophs (e.g. Dubois *et al.* 2000), which synthesize and secrete gonadotropins to regulate testis physiology.

Recent work in mammals has shown that the neuropeptide kisspeptin (KiSS1) and its receptor (GPR54) provide crucial, stimulatory input to the Gnrh neurons for the onset of puberty and for the adult functioning of the Gnrh-gonadotroph-gonadal axis (Shahab *et al.* 2005). The Kiss1/Gpr54 system is also present in fish, has been implicated in pubertal maturation (Parhar *et al.* 2004; Felip *et al.* 2008; Filby *et al.* 2008; Kitahashi *et al.* 2009; Li *et al.* 2009) and, interestingly, in the mediation of photoperiod signaling for seasonal reproduction (Revel *et al.* 2006). Unfortunately, there is no information yet on the Kiss1/Gpr54 system in Atlantic cod. However, the exquisite sensitivity of this species to photoperiod manipulation renders it an excellent model in this regard for future research. As for now, we assume that the 'decreasing day length' signal results – possibly via a Kiss1/Gpr54/Gnrh cascade – in activating the pituitary Fsh synthesis and release.

At this point, the question of predicting gonadotropin release based on quantifying pituitary mRNA amounts requires attention. One aspect refers to the fact that Fsh and Lh are heterodimeric glycoproteins consisting of a hormone-specific β-subunit but that share a common α-subunit. Only the β-subunits were quantified in the present study, since α-subunit availability is not a limiting factor for the gonadotropins availability in different vertebrates (eg. African catfish, Schulz *et al.* 1995; bullfrog, Tanaka *et al.* 1991). Secondly, while it appears reasonable to assume that the amount of mRNA reflects gonadotropin synthesis, this is not necessarily the case for gonadotropin release. In fish, data on pituitary and plasma levels of Fsh and Lh during different stages of reproductive development are only available for salmonid fish

(Campbell *et al.* 2003; Gomez *et al.* 1999). In these studies, pituitary contents and plasma Fsh levels changed in a similar manner, except for post-spawning fish, when pituitary levels increased and plasma levels decreased in both salmon and trout. Similar to the situation in mammals, in these studies Fsh plasma levels were higher than Lh levels. In mammals, the higher basal release of Fsh than of Lh reflects the observation that most of the Fsh secretion occurs in the constitutive release pathway, while Lh release nearly exclusively occurs via the regulated pathway (Jablonka-Shariff *et al.* 2008). Hence, as regards *fshb* mRNA levels in cod pituitary, we will assume that they also reflect the release of Fsh and as regards *lhb* mRNA, on the other hand, the levels will be considered as reflecting pituitary Lh amounts, while changes in circulating androgen levels, a classic domain of Lh bioactivity, will be considered in addition in discussions on the biological activity of circulating Lh.

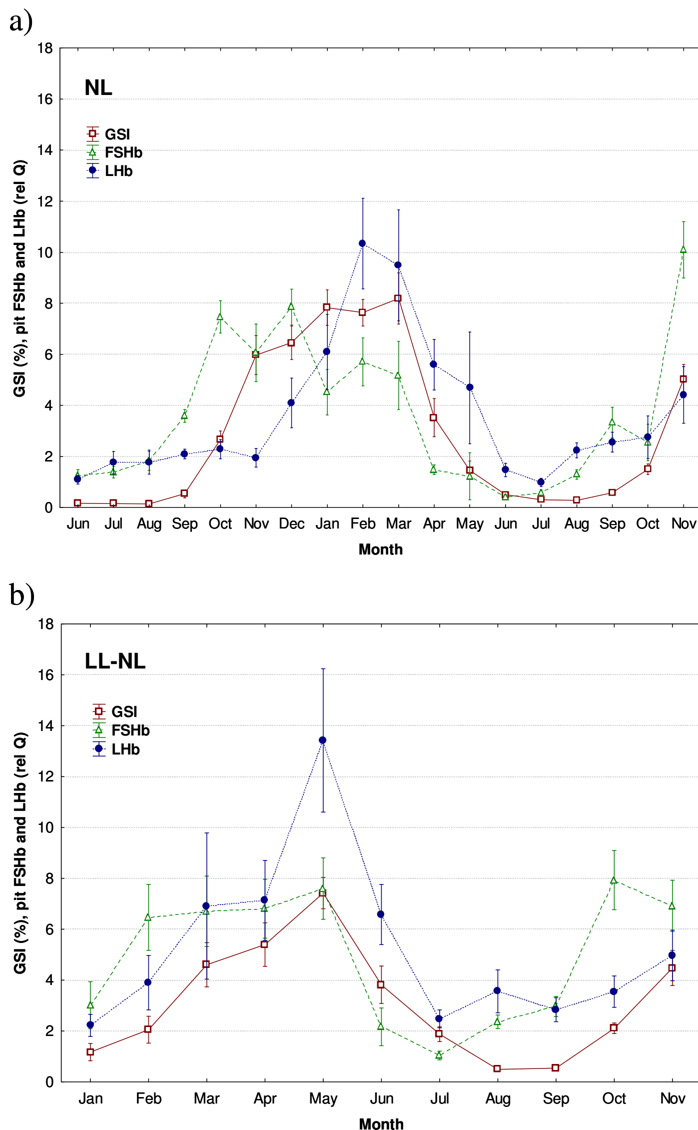


Figure 4. Gonado-somatic index (**GSI**; %) and pituitary *fshb* and *lhb* relative quantities (**rel Q**). a) In fish from the normal light (**NL**) group; b) in fish from the group moved from constant light to normal light (**LL→NL**). Data from July 2004 to November 2005 in NL group and from January to November 2005 in the LL→NL group.

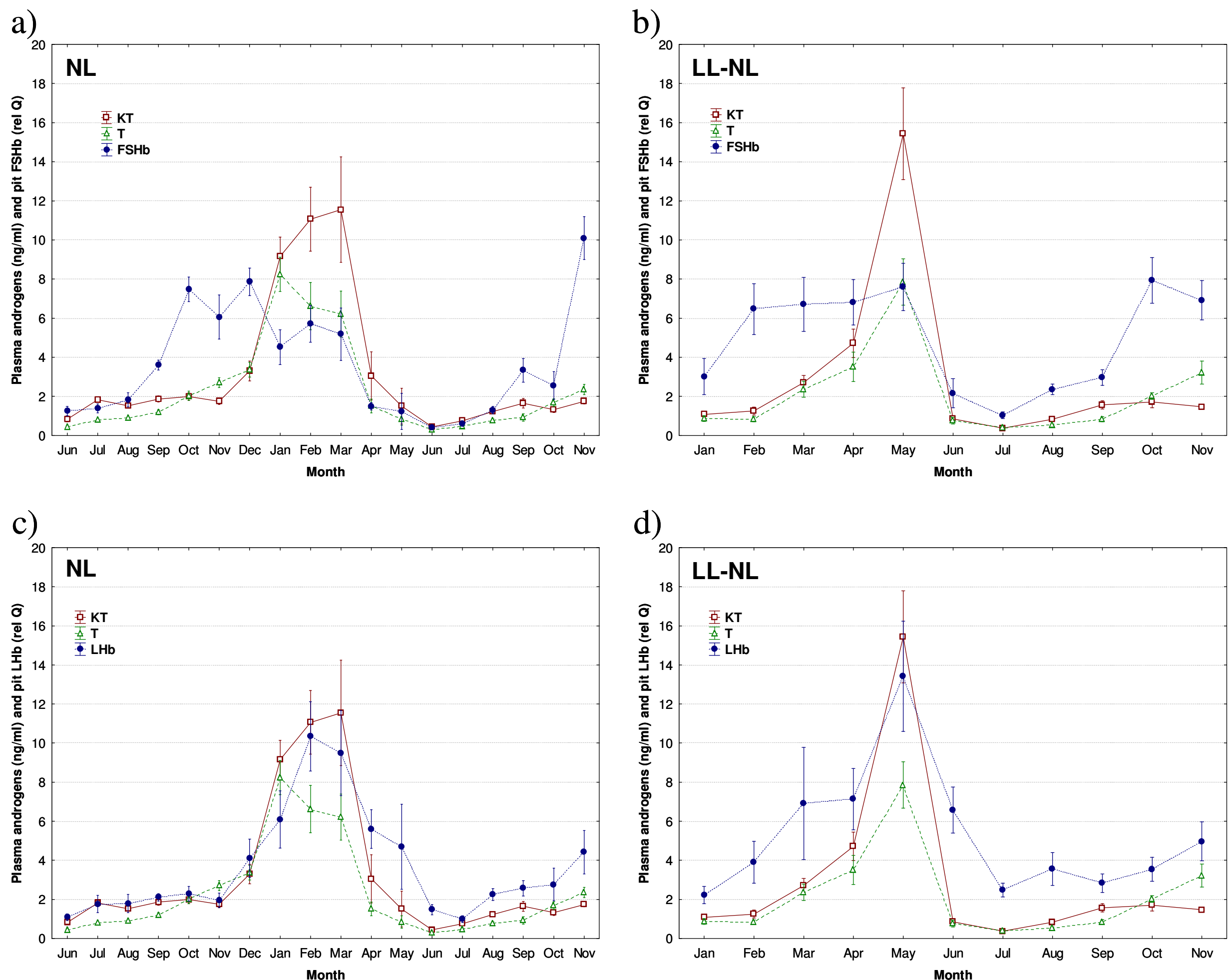


Figure 5. Androgen plasma levels and pituitary gonadotropin sub-unit expressions. *fshb* in the top and *lhb* in the bottom. a) and d) fish from the normal light (**NL**) group; b) and d) fish from the group moved from constant light to normal light (**LL→NL**). **T**: testosterone; **KT**: 11-ketotestosterone. Data from July 2004 to November 2005 in the NL group and from January to November 2005 in the LL→NL group.

The pituitary levels of *fshb*, but not of *lhb*, mRNA increased during the start of cod testis maturation in the two cycles, 2004 and 2005, suggesting that an Fsh-specific mechanism was active. In mammals, both Fsh and Lh are produced by the same pituitary cell type, while in fish two different gonadotroph cell types exist (Naito *et al.* 1993). If photoperiod signals stimulated Gnrh release (as discussed above), one possibility to explain an Fsh-specific response could be assuming that at certain stages Fsh-producing, but not Lh-producing, gonadotrophs express Gnrh receptors. Elevated levels of pituitary and plasma Fsh, but not Lh, have also been observed in rainbow trout (Gomez *et al.* 1999) and Chinook salmon (Campbell *et al.* 2003) at the start of pubertal testis growth. Examples for Fsh effects on Sertoli cells are the release of growth factors that modulate germ cell proliferation and differentiation, for example the balance between self-renewal and differentiation of spermatogonial stem cells in mice (Tadokoro *et al.* 2002), the entry of type B spermatogonia into meiosis in the newt *Cynops pyrrhogaster* (Yazawa *et*

al. 2002). In summary, we hypothesize that Fsh-mediated signaling triggers the start of spermatogenesis and also supports the entry of germ cells into meiosis. This assumption is in line with the observation that exposing animals to LL during the summer/fall period prevents the rise in *fshb* mRNA expression and testis growth. Moving animals from LL to NL conditions, on the other hand, induced an out-of-season increase in *fshb* mRNA levels, leading to an out-of-season testis growth.

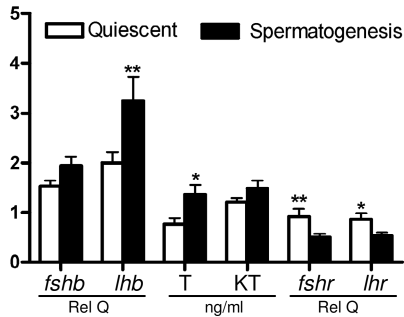


Figure 6. Graph showing differences in gene expression and androgen plasma levels in fish from the constant light group showing either developing spermatogenesis or quiescent testis. Asterisks represent significant differences (*p < 0.05 and **p < 0.01)

Despite the large changes in testis weight and cellular composition during the cycle, there are only minor changes, compared to *lhr* mRNA, in the testicular *fshr* mRNA levels. The repeated observation may be significant that relatively high levels of expression were measured in quiescent testes (July 2004 and July 2005) that decreased towards November in the period when the main testis weight gain took place. Also when testis development in the LL group was induced by the switch to NL conditions, high GSI levels were reached in March when *fshr* mRNA attained its lowest values. As in other vertebrates, *fshr* is expressed in Sertoli cells in fish testis (Schulz *et al.* 2009). The cellular composition changes during spermatogenesis and the germ/somatic cell ratio increases per cyst (Billard 1969b; Matta *et al.* 2002; Schulz *et al*. 2005; Leal *et al.* 2009). We therefore assume that a dilution of Sertoli cell-derived mRNAs took place during rapid testis growth. When expressing *fshr* mRNA levels in the same way as in the present study, Sambroni et al. (2007) also reported a decrease in testicular *fshr* mRNA levels in rainbow trout during the start of pubertal testis growth. Kusakabe *et al*. (2006) and Maugars and Schmitz (2008), on the other hand, who included testicular weight gain as a factor to estimate *fshr* gene expression, reported slightly (rainbow trout) or strongly (Atlantic salmon) increasing *fshr* mRNA levels during testis maturation. In summary, we propose that testicular *fshr* expression has a strong constitutive component while the relatively small changes in expression levels may also be related to changes in the tissue composition.

Recent reports on the cellular localization of *fshr* mRNA have shown that besides intratubular Sertoli cells, also interstitial Leydig cells express the *fshr* gene in Japanese eel (Ohta

et al. 2007) and African catfish (García-López *et al.* 2009), an observation that explains the steroidogenic activity of Fsh in a number of teleost species (e.g. eel, Kamei *et al.* 2003; Ohta *et al.* 2007; catfish, Vischer *et al.* 2003; Zmora *et al.* 2007; salmon, Planas and Swanson 1995). In the Atlantic cod, we recorded extended periods with elevated *fshb* mRNA levels (September – November in 2004 and 2005 in the NL group, February and March 2005 in the LL→NL group) while plasma androgen levels either remained low (KT in all months mentioned) or increased slowly (T in October and November in the NL group). Also in three-spined stickleback and rainbow trout, plasma androgen levels are low during the period of spermatogenesis (Andersson *et al.* 1988; Kusakabe *et al.* 2006). Future work will have to elucidate the presence of Fshr on the steroidogenic Leydig cells in Atlantic cod testis, and the possible relation to the slowly increasing plasma T levels. After all, these rising androgen levels seem sufficient to activate the androgen receptor (see Chapter 5) expressed by Sertoli cells enveloping proliferating spermatogonia and might support androgen-dependent processes during spermatogenesis, as it has been described for example in Japanese eel (Miura and Miura 2001).

The rise in androgen plasma levels in male cod initiates slowly from October to December, until a steep rise of both T and KT between December and January (Chapter 3). Androgen treatment during full spermatogenesis reduced the pituitary Fsh levels in Atlantic salmon parr (Borg *et al.* 1998) and sea bass (Mateos *et al.* 2002), and reduced circulating Fsh levels in Coho salmon (Dickey and Swanson 1998). Therefore, we assume that the steep increase in circulating androgen levels observed after the winter solstice may be responsible for the partial down-regulation of *fshb* mRNA levels that, in combination with a negative, androgen-mediated feedback on Fsh release, may have significantly reduced Fsh-signaling. In this context, it is tempting to speculate that the cessation (from February onwards) of spermatogonial proliferation in the NL group reflects waning Fsh stimulation.

In February, meiosis, spermiogenesis, and spermiation were completed and the spawning condition was attained in the NL group. These late maturational processes in general are considered to be more dependent on Lh than on Fsh in fish (Swanson 1991; Gomez *et al.* 1999; Yaron *et al.* 2003). As shown in Fig. 5c, the changes in plasma androgen levels, in particular with regard to KT, and pituitary *lhb* mRNA levels closely paralleled each other. Studies in different fish species have shown that castration results in a decrease of pituitary Lh protein and/or *lhb* mRNA levels (Hellqvist *et al.* 2008; Borg *et al.* 1998; Cavaco *et al.* 2001). These studies also showed that replacement with aromatiseable androgens, such as T, had strong stimulatory effects on the pituitary Lh amount that depended on the conversion of T to estrogen (Borg *et al.* 1998; Rebers *et al.* 2000), but that part of the stimulatory effect can also be attributed to non-aromatiseable KT. These observations can be bundled into the assumption that the

increased steroidogenic capacity of Leydig cells, resulting in steeply increasing plasma androgen levels, and the concomitant increasing pituitary *lhb* mRNA levels are connected to each other in a positive feedback loop that drives testis development towards the spawning condition.

Examining the *lhr* mRNA levels revealed that changes in the expression of the receptor gene followed *lhb* expression with a lag-time of one month, suggesting that changes in receptor expression were secondary to changes in ligand expression. This was observed in the NL and also in the LL→NL group. Although dilution of presumably Leydig cell-derived mRNA during rapid testis growth might be relevant for *lhr* mRNA as well, this was not observed, leading us to assume that *lhr* gene expression was strongly up-regulated in spawning fish.

How is the Lh/androgen loop initiated and what conditions result in the stop of its function? For the first part of the question, the comparison of the patterns between the NL and the NL→LL treatment groups is very interesting. In the NL→LL group, we observed a sudden increase, as opposed to a more gradual increase in the NL group, of both the *lhb* and *lhr* mRNA levels. This suggests that an increasing as well as an abruptly attained long photoperiod, when following short days that allowed testis development to proceed, triggered an up-regulation of the activity of the Lh-dependent signaling system. Interestingly, Atlantic cod seem able to differentiate between, and respond accordingly, sudden (NL→LL) and gradual (NL) increases in day-length. The observation of increasing testicular *lhr* mRNA levels during the development towards the spawning season with concurrent increases in androgen plasma levels and in the expression of steroidogenic enzyme genes (eg. *star* and *cyp17a* mRNAs) has been observed in rainbow trout maturing under NL conditions (Kusakabe *et al.* 2006). Also in maturing Atlantic salmon parr, pituitary *lhb* and testicular *lhr* mRNA levels, and plasma androgen levels increased concomitantly (Maugars and Schmitz 2008). However, to our knowledge, there are no data available to compare the changes induced by exposure to different photoperiod regimes in male fish, except for sea bass that also showed LL-induced inhibition of pituitary gonadotropin subunit contents (Rodriguez *et al.* 2005).

GSI and plasma androgen levels (Almeida *et al.* 2009), as well as *lhb*, *lhr*, and – at an intermediate level –*fshb* mRNA levels all stayed in an up-regulated state in February and March in the NL group, before a coordinated down-regulation of all parameters (except for the *lhr* mRNA levels, where we encountered a “still high” and an “already low” subgroup) occurred in April. In the NL→LL group, this down-regulation already occurred in March as regards *lhb* and *lhr* mRNA, and as early as February as regards *fshb* mRNA. Therefore, we assume that, in analogy to the line of thought developed with respect to the start of the positive feedback loop, its termination may be triggered by the increasing photoperiod towards the summer. We

propose that the summer-typical long photoperiod is incompatible with an elevated level of activity of the neuroendocrine circuits merging onto the Gnrh neurons innervating the pituitary gonadotrophs. After all, continuing experimentally the long photoperiod over the summer solstice by exposing animals to LL conditions prevents the start of testis development otherwise triggered by the decreasing day length in late summer. It would, therefore, appear that in Atlantic cod the signal "long photoperiod" can have different effects, depending on the photoperiod history of the animals: coming from a short photoperiod, Lh-dependent signaling becomes highly activated, while coming from long days, a continued exposure to a long photoperiod would prolongate an overall inhibitory condition. A decreasing photoperiod, on the other hand, would specifically allow activation of Fsh-dependent signaling.

Submitting fish to LL suppressed the main increases in gonadotropin and gonadotropin receptor gene expression and blocked testis growth. Still, in about 1/3rd of the LL-exposed males, a quantitatively and qualitatively limited spermatogenesis took place (Chapter 3). The *fshr* expression in the LL subgroup showing some spermatogenic activity was lower than in quiescent males. This would be compatible with the situation in fish from other groups, where receptor mRNA dilution in context with the increasing number of germ cells has been discussed already (see above). Fish showing some spermatogenic activity had higher plasma T levels than quiescent fish, and although these levels were still lower than in maturing males of the NL group, the elevated T levels may have been responsible for the elevated pituitary *lhb* mRNA levels found in this subgroup. Interestingly, the *fshb* mRNA levels were not different, which might explain the fact that only a few cysts were recruited into spermatogenesis.

In summary, we quantified increasing *fshb* mRNA expression in pituitaries of cod entraining pubertal testis development, while *fshr* seemed to be constitutively expressed in the gonads during the annual cycle. At later stages of spermatogenesis, prior to and during spawning, *lhb*, followed one month later by its cognate receptor, was up-regulated, in parallel with androgen plasma levels. It appears that Atlantic cod BPG axis can respond in two manners when submitted to a long photoperiod, depending on the photoperiod history before the exposure to constant light.

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Supplemental Table Summary of the statistical comparison of monthly mean gene relative expression between the light treatment groups. *

	2004						2005										
	J	**A**	**S**	**O**	**N**	**D**	**J**	**F**	**M**	**A**	**M**	**J**	**J**	**A**	**S**	**O**	**N**
fshb																	
NL		b	b	b	b	b	b	b	a,b	a	a	b	b	a,b		a	b
LL		a	a	a	a	a	a	a	a	a	a	a	a	a,b		a	a
NL-LL							b	a	c	a	a	a	a	b		a	a
LL-NL							a,b	b	b	b	b	a	b	a		b	b
lhb																	
NL							b	b		a	a	a	b				
LL							a	a		a	a	a	a				
NL-LL							c	b		b	a	a,b	a				
LL-NL							a,b	a		a	b	b	a				
fshr																	
NL			b			b			b				b	b			a
LL			a			a			a				a	a			a,b
NL-LL									a				a	a,b			b
LL-NL									a				a,b	a,b			a
lhr																	
NL							a		b		b	a					
LL							a		a		a,b	a					
NL-LL							b		a		a,b	a,b					
LL-NL							a		a		a	b					

*Data from July 2004 to November 2005; columns labeled with the same latter do not differ significantly (P> 0.05).

5

Cloning, Pharmacological Characterization and Expression Analysis of Atlantic Cod (*Gadus morhua*) Androgen Receptor

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ABSTRACT

Androgen receptors are important mediators of androgen action in developmental, metabolic and reproductive processes in both male and female vertebrates. In the present study, we report the cloning of a cDNA coding for a nuclear androgen receptor protein (Ar) from Atlantic cod testis. Maternally deposited *ar* mRNA was detected in cod embryos one day after fertilization and expression decreased to very low levels at 13 days after fertilization, before gonadal sex differentiation. In adult fish, *ar* was expressed in all seven tissue types tested, with sexually dimorphic, male-biased expression only in gonadal tissue. Testicular *ar* expression was quantified during the first (pubertal) reproductive season and showed maximum levels when full spermatogenesis (coinciding rapid spermatogonial proliferation, meiosis, spermiogenesis and spermiation) was ongoing and at the beginning of spawning. Cod Ar protein, transiently expressed in mammalian (HEK 293T) cells, showed specific androgen binding as well as androgen-dependent transactivation of the androgen responsive MMTV promoter. Examining plasma levels of six androgens at different stages of the annual reproductive cycle demonstrated that the high affinity ligands testosterone and 11-ketotestosterone were present in significant concentrations at all stages, that testosterone levels started to increase earlier than 11-ketotestosterone, and that both androgens showed maximum levels in mature males. The Atlantic cod *ar* mRNA expression pattern suggests Ar functions during early stages of ontogenesis, during intermediate stages such as sex differentiation, as well as during adult functioning of the adult testis, i.e. spermatogenesis and steroidogenesis.

INTRODUCTION

Androgens play essential roles during development and in adult vertebrates; their specific biological activities, in particular as regards the reproductive system, have been the subject of many studies. In teleost fish, the largest vertebrate group close to the basis of the phylum, androgens have important functions and are involved, for example, in sex differentiation (Kobayashi *et al*. 1991; Baroiller and Guiguen, 2001), oocyte development (Rohr *et al*. 2001; Lokman *et al*. 2007; Kortner *et al*. 2009), spermatogenesis (Miura *et al*. 1991; Cavaco *et al*. 2001), Leydig cell steroidogenesis (Cavaco *et al*. 1999; Schulz *et al*. 2008), secondary sexual

characters and reproductive behavior (Stacey and Kobayashi, 1996; Pall *et al*. 2002a,b). Next to testosterone (T), an androgen found in all vertebrates, androgens with an oxygen function at C-atom 11, such as 11-ketotestoterone (KT), are also typically found in teleost fish (Borg, 1994).

Androgens exert their effects by modulating the expression of target genes via their cognate receptor. The androgen receptor (Ar) belongs to the nuclear receptor family of proteins and functions as a ligand-activated transcription factor influencing gene expression by binding to response elements in regulatory regions of androgen-responsive genes (Evans, 1988; Jenster *et al*. 1992; Mangelsdorf *et al*. 1995). Hence, nuclear receptors possess a highly conserved DNA-binding domain (DBD), a moderately conserved ligand-binding domain (LBD), and an evolutionary divergent transactivation domain (TAD; McEwan *et al*. 2007).

The *ar* cDNA has been cloned from several vertebrate species and usually one type of functional androgen receptor has been identified per species. In some cases however, particularly in fish, additional *ar* forms have been characterized, such as in rainbow trout (*Oncorhynchus mykiss*; Takeo and Yamashita, 1999), mosquitofish (*Gambusia affinis*; Ogino *et al*. 2004; Sone *et al*. 2005), *Astatotilapia burtoni* (Harboot *et al*. 2007), Nile tilapia (*Oreochromis niloticus*), Japanese eel (*Anguilla japonica*; Ikeuchi *et al*. 1999; Todo *et al*. 1999) and three-spined stickleback (*Gasterosteus aculeatus*; Olsson *et al*. 2005). In addition to these molecular data, different Ar forms have been characterized biochemically in two fish species (Sperry and Thomas, 1999a,b) based on their binding affinities for different androgen and differential tissue distributions in gonad and brain. While the presence of additional *ar* genes is discussed in context with the genome duplication that all fish have undergone (Jaillon *et al*. 2004), there are also species that went through an additional genome duplication, such as the zebrafish, in which only a single Ar variant has been found (de Waal *et al*. 2008; Douard *et al*. 2008; Hossain *et al*. 2008).

The Ar protein is expressed in several tissues in both sexes, and *ar* mRNA is present at early stages of ontogenesis in different (developing) tissues (e.g. Blazquez and Piferrer, 2005 and Gorelick *et al*. 2008), indicating a broad range of Ar-mediated biological activities. Although the presence of Ar protein has been reported in germ cells (Vornberger *et al*. 1994; Merlet *et al*. 2007), most studies in adult mammals and teleosts reported that germ cells in the testis are devoid of Ar protein (Sharpe, 1994; Bremner *et al*. 1994; Shan *et al*. 1997; Weinbauer and Nieschlag, 1997; McLachlan *et al*. 2002; de Waal *et al*. 2008). Moreover, germ cell-specific androgen receptor knockout mice presented normal spermatogenesis (Tsai *et al*. 2006) and there is no requirement of direct action of androgens on germ cells during the process of spermatogenesis (Johnston *et al*. 2001). Nonetheless, androgens are strictly required for spermatogenesis and exert indirect effects via cell-cell communication with somatic cells,

primarily Sertoli cells and peritubular myoid cells (Roberts and Zirkin, 1991; Chen *et al*. 1994; Wang *et al*. 2009). In mice, androgen action on Sertoli cells allows germ cells to complete meiosis (de Gendt *et al*. 2004; Chang *et al*. 2004; Tsai *et al*. 2006) and spermatid differentiation (Holdcraft and Braun, 2003). Leydig cells also express the Ar protein (Shan *et al*. 1995) and androgen/Ar signaling has an autocrine regulatory function to modulate the steroidogenic function of Leydig cells (Chang *et al*. 2004). Studies on the identification of androgen target cells in teleost testes are scarce. However, two recent studies showed specific *ar* mRNA expression in Sertoli cells enveloping early spermatogonia (de Waal *et al*. 2008; Almeida *et al*. 2009), suggesting that – similar to the situation in mammals – *ar* mRNA expression differs among Sertoli cells contacting spermatogenic cells in different developmental stages.

Atlantic cod is a species with an increasing economic value world-wide. With continuous fluctuations in the supply from fisheries and relatively good suitability for aquaculture, the interest in intensive production of cod has increased over the recent past. However, as an exclusively marine species, cod are able to spawn in the net pens when they reach maturation before harvest time/size. As in many species introduced in aquaculture, the problem of early maturation is prevalent in males, so that possibilities are sought to generate all-female populations. Early maturation and consecutive spawning entails risks of genetic impacts on wild populations (Jørstad *et al.* 2008) and is accompanied by losses in flesh quality and body growth (Taranger *et al*. 2006). Clearly, early puberty is a non-desired event in farmed fish and the most used tool to prevent sexual maturation in cod is to change the photoperiod regime. Respective studies indicated that the pubertal increase in testis weight and androgen plasma levels are prevented by exposure to constant light (Norberg *et al*. 2004; Almeida *et al*. 2009). Investigating the androgen signaling system may contribute to understanding the biological basis of sex differentiation on the one side and of photoperiod-inhibited testis development on the other. To this end, we decided to clone and functionally characterize Atlantic cod *ar* cDNA, and to quantify *ar* expression at different stages of cod ontogenesis. To obtain information on the natural ligands for the cod Ar, we quantified different natural androgens in plasma pools obtained from males at different stages of the reproductive cycle.

MATERIAL AND METHODS

Molecular Cloning of Cod *ar* cDNA

Cod testis total RNA was reverse transcribed with random hexamers using the Superscript II preamplification System (Invitrogen, Breda, The Netherlands), according to the

manufacturer's instructions. Poly(A)-rich cod testis RNA was prepared using Dynabeads-oligo dT_{25} (Dynal A.S., Oslo, Norway) and 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 cod *ar* cDNA sequence, 2 µl random hexamer-primed cod testis cDNA was used as template in a PCR with degenerate primers 2197 and 2198 (Supplemental Table 1), corresponding to highly conserved amino acid sequences (LTCGSCKVFFK and FLCMKALLLF) found in known Ars. PCR was carried out in a 50 µl volume using the Advantage 2 PCR system (Clontech) in a Perkin-Elmer 2400 thermal cycler (Applied Biosystems, Foster City, CA, USA). PCR was performed under the following cycling conditions: denaturation at 94^0C for 30 s, followed by 35 cycles of 94^0C for 15 s, 59^0C for 15 s and 72^0C for 50 s. DNA fragments of approximately 750 bp were subcloned into pcDNA3.1/V5-His TOPO vector (Invitrogen) and plasmid DNA of 5 clones was prepared for DNA sequence analysis.

To isolate the 5′- and 3′-ends of the cod *ar* cDNA, gene-specific primers (*i.e.* primers 2230 and 2237; Supplemental Table 1) – based on the consensus nucleotide sequence of the 5 clones, each containing the above mentioned 750 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 2231 and 2238; Supplemental Table 1) in combination with a nested universal primer (NUP), respectively. Both the UPM and NUP were supplied with the SMART RACE cDNA amplification kit (Clontech). All RACE reactions were carried out according to the manufacturer's instructions in a Perkin-Elmer 2400 thermal cycler (Applied Biosystems) using Advantage 2 polymerase (Clontech). RACE products were subcloned into pcDNA3.1/V5-His TOPO vector (Invitrogen). The open-reading frame of the cod *ar* was PCR amplified using primers 2269 and 2270 (Supplemental Table 1), subcloned into pcDNA3.1/V5-His TOPO vector (Invitrogen) and checked for their correct orientation. DNA sequence analyses were performed using Dye Terminator cycle sequencing chemistry (all from Applied Biosystems).

Sequence Analysis

Deduced full-length Ar sequences were obtained from the GenBank database and used for amino acid comparisons and phylogenetic analysis (accession numbers in Supplemental Figure). The alignment of these Ar proteins and the calculations of the percentages similarity and percentages divergence were performed using the Megalign program of the Lasergene 7.1 software package (DNASTAR Inc., Madison, WI, USA) according to the Clustal W (PAM250) algorithm (Higgins and Sharp, 1989). A phylogenetic tree was constructed from the aligned

sequences by the Neighbor-Joining method (Saitou and Nei, 1987), using the three zebrafish (*Danio rerio*) estrogen receptors to form an out group.

Cod Androgen Plasma Levels

The reproductive cycle of male Atlantic cod can be divided in four main developmental stages: 1- immature or quiescent testis (June/July), in which only single/type A spermatogonia are observed; 2- early maturing testis, in which spermatogonia rapidly proliferate, increasing cyst number and size (August/September); 3- testis in full spermatogenesis, in which spermatogonial proliferation is still ongoing but also meiosis, spermiogenesis and spermiation (October - January); and finally, 4- spawning testis, in which spermatogenesis is completed with free spermatozoa observed in the testis tubular lumen and sperm is hydrated and can be released by slight pressure on the abdomen (February - April). Based on this classification, we identified seven animals per stage and pooled their plasma to generate samples representing these four stages. Steroids were extracted from the plasma pools as described in Hyllner *et al*. (1994), dissolved in few droplets of dichloromethane-methanol (9:1) and applied to a thin layer chromatography (TLC) plate. The TLC plate was developed three times in toluene-cyclohexane (1:1) to separate apolar compounds present in the extracts from the steroids. Thereafter, the plate was developed in dichloromethane-ethanol (95:5) to separate the steroids of interest from each other. Localization of the steroids on the TLC plate, their elution from the silica gel, and subsequent androgen quantification by specific radio-immunoassay were carried out as described previously (Schulz 1985). The following steroids were quantified: testosterone (4-androsten-17β-ol-3-one, T), 11-ketotestosterone (4-androsten-17β-ol-3,11-dione, KT), androstenedione (4-androstene-3,17-dione, A), 11-ketoandrostenedione (4-androstene-3,11,17-trione, KA), 11β-hydroxytestosterone (4-androsten-11β,17β-diol-3-one, OHT) and 11β-hydroxyandrostenedione (4-androsten-11β-ol-3,17-dione, OHA). Legal restrictions did not allow the use of radioactive tracers at the site of extraction and TLC to monitor losses during the procedure. However, comparing the present results (see Fig. 1a) with the previous quantification of T and KT by ELISA in a large number of individual samples from males of the same experiment, we estimate that we recovered ~50% of T and KT in the present study. Assuming a similar recovery for all six steroids, the data presented in Fig. 1a are probably underestimated by 50%.

Ligand-binding Assay

Transactivation assays were performed following the protocol used by de Waal *et al*. (2008). For this purpose, HEK 293T cells were co-transfected with 250 ng of cod *ar* expression

plasmid, 7 μg of MMTV-Luc plasmid and 1 μg of EGFP (enhanced green fluorescent protein) plasmid. For the transfection, the cells were incubated in phenol-free medium containing 10% v/v charcoal-stripped serum. Each assay was conducted in duplicate, three independent transfections were carried out, and the data were pooled for statistical analysis.

Saturation and Ligand-competition Assays

For saturation ligand-binding assays, $[^3H]$-testosterone ($[^3H]$-T) (specific activity 77.0 Ci/nmol; PerkinElmer, Boston, USA) was used as tracer, as described previously (de Waal *et al*. 2008). Four μg of cod *ar* cDNA (and 8 μg of carrier plasmid) were used for the transfections. The dissociation constant (K_d) was assessed by non-linear regression using GraphPad Prism Software (San Diego, USA). As a control, the same procedure was carried out using empty vector.

To determine the affinity of other androgens to the cod Ar, a ligand competition assay was developed as described in de Waal *et al*. (2008). Increasing concentrations (0.1 nM to 0.1 mM) of natural androgens (KT, A, KA, OHA, OHT) were used to compete with a fixed concentration (2 nM) of $[^3H]$-T. The EC_{50} values were calculated with non-linear regression and K_i values calculated using a dose-response curve of non-labeled testosterone (included in each experiment) as follow: K_i = (EC_{50} steroid/ EC_{50} T) x Kd $[^3T]$-T. The experiments described above were carried in duplicates and three independent transfections were carried out for each type of experiment.

Cod *ar* mRNA Expression During Larval Development and in Adult Tissues

Total RNA was isolated from a pool of the following material: embryos 24 hours and 4 days after fertilization; larvae of 5 to 6 mm total length, just before first feeding. Total RNA was extracted from individual larvae, after head removal, from 6.3 mm to 57 mm full-length (n = 2 - 21); the sex of the larvae was unknown, as a genetic sex marker is not yet available for Atlantic cod. In adult cod, total RNA was isolated from head kidney, gill, spleen, heart, intestine, liver and gonad of three males and three females.

To study possible effects of photoperiod manipulation on testicular *ar* expression in cod, we extracted total RNA from testis tissue of animals kept under natural light (NL), allowing normal pubertal development, or kept under continuous light (LL), a condition known to inhibit pubertal testis growth. Samples (n = 4 per month and photoperiod condition) were collected monthly from June – November and again in February. Under NL conditions, testes were quiescent (June – July), spermatogonial proliferation had started (August-September), full spermatogenesis was ongoing with all stages of germ cells including spermatozoa (October and

November), or males were in spawning condition with testes filled with sperm and occasionally quiescent spermatogonia (February). Under LL conditions, independent of the month, testes were either quiescent or showed restricted spermatogenic activity with all germ cell stages present - including spermatozoa. In both treatment groups, the gonado-somatic index (GSI) was determined, expressing testis weight as percent of body weight.

All RNA extractions were performed by the acid phenol-guanidine thiocyanate method after tissue homogenization in the FastPrep tube containing Lysing Matrix D ceramics beads. Random-primed cDNA was synthesized from 500 ng RNA using the Reverse Transcriptase Core Kit (RT-RTCK-05, Eurogentec, Belgium).

Primers 2655 and 2656 (Supplemental Table 1) were designed to detect cod *ar* mRNA using real-time, quantitative PCR (qPCR). The specificity and efficiency of this *ar* primer set were validated with qPCR on serial dilutions of cod testis cDNA. All qPCRs for the cod *ar* were performed in 20 µl reactions, containing 10 µl Power SYBR Green PCR Master mix (Applied Biosystems), 0.9 µM forward primer (2655), 0.9 µM reverse primer (2656) and 5 µl diluted (1:10) cDNA. For each sample, two PCR reactions were performed and then averaged. Data were analyzed by 7900HT Fast Real-Time PCR System (Applied Biosystems). Quantification of the transcripts were normalized using cod elongation factor-1α (*ef1a*) as a reference gene (Mittelholzer *et al*. 2007). In early stages, the average of all quantitative values for the embryos and larvae was used as a calibrator, whereas the average of testis was the calibrator for adult tissue analysis. For comparing testicular *ar* transcripts in the photoperiod treatment groups, the average of quantitative *ar* values of fish from the NL group was used as a calibrator.

Statistical differences in relative *ar* mRNA expression in larvae of different lengths (embryos were pooled, not allowing statistical analysis) and in testis of NL and LL groups during different months, as well as the GSI, were assessed by analysis of variance of LOG-transformed values, followed by Newman-Quews post-test. For the tissue screen data (female *versus* male *ar* expression) and monthly comparison between NL and LL groups (*ar* expression and GSI) unpaired t test was used. Data are presented as mean ± SE and differences were considered as significant when $P \leq 0.05$.

RESULTS

Cod *ar* cDNA cloning

As a first step to isolate the cod *ar* cDNA, we designed a set of degenerate primers (*i.e.* primers 2197 and 2198; Supplemental Table 1), targeting highly conserved *ar* sequences, and

obtained a ~750 bp PCR product that was gel purified, extracted and cloned. Five clones were sequenced, all providing the same partial *ar* cDNA sequence, while no evidence for the presence of a second cod *ar* cDNA sequence was found. New cod *ar*-specific primers (2230, 2231, 2237 and 2238; see Supplemental Table 1), based on the single consensus nucleotide sequence of the cloned RT-PCR products, were used in RACE reactions in order to isolate the 5′- and 3′-ends of the cod *ar* cDNA.

Combining the partially overlapping sequences of the 5′- and 3′-RACE products yielded a full-length cod *ar* cDNA sequence with 2157 nucleotides in the open-reading frame. The open-reading frame was PCR amplified using primers 2269 and 2270, and subcloned in the correct orientation into the pcDNA3.1/V5-His-TOPO expression vector. Sequence analysis of several clones revealed identical *ar* sequences as those obtained in the 5′- and 3′-RACE clones, and the sequence of the cod *ar* open-reading frame was deposited at GenBank with accession number FJ268742.

Cod Ar Sequence and Phylogenetic Analysis

Conceptual translation of the open-reading frame predicted a 718-amino acid protein with an estimated molecular mass of 79.53 kDa. The protein displayed the typical features and domain structure of members of the nuclear receptor family, in particular of Ars. As other Ars, the cod Ar protein showed three domains (Supplemental Fig. 1a): the transactivation domain (TAD), the DNA-binding domain (DBD) and the ligand-binding domain (LBD). Comparison of the cod Ar sequence with other vertebrate Ar proteins revealed that the full-length cod Ar protein showed the highest level of sequence similarity to the mosquitofish (*Gambusia affinis*) and tilapia (*Astatotilapia burtoni*) Ars (62.7%) and the lowest homology (37.8%) to mouse (*Mus musculus*) and pig (*Sus scrofa*) ARs.

The amino acid alignments were also used for constructing a phylogenetic tree using the Neighbor-Joining method. The tree showed three main clades. The largest grouped most of known teleost Ars, a second included AR from mammals and birds, and the third, in which cod Ar was present, grouped the second type of Ar described in some teleosts species from three different families: tilapia (Cichlidae), mosquitofish (Poeciliidae) and medaka (Adrianichthyidae) (Supplemental Fig. 1b).

Plasma Androgen Profile

The levels of OHT varied between 0.7-1.2 ng/ml without showing a clear relation to the reproductive stage (Fig. 1a). A common feature of all 5 other androgens was that they showed relatively low levels in immature and early maturing fish and also during full spermatogenesis,

while the main increase was observed in the plasma pool from spawning males. In spawning fish, T, OHA, and KT levels increased to ~2-4 ng/ml, while KA and A were found at higher levels of ~9-14 ng/ml. The structure of the six androgens and their position in the biosynthetic pathway is sketched in Fig. 1b.

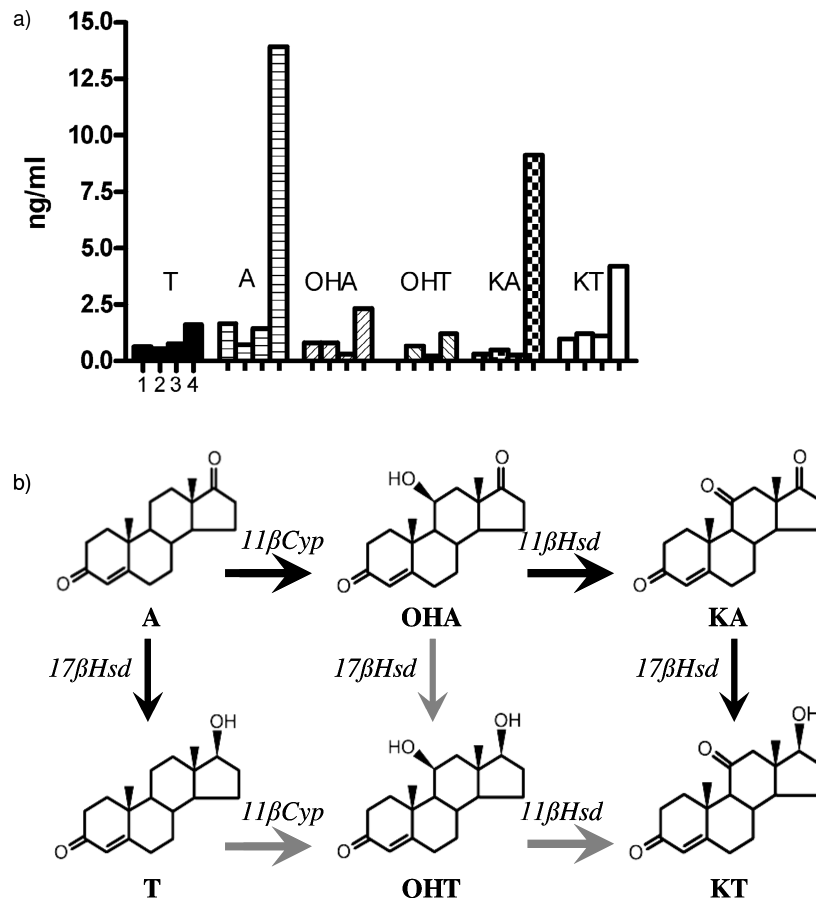

Figure 1. Cod androgen plasma levels and steroidogenic pathway. a) Androgen plasma levels analyzed by RIA using extracted plasma pool samples (n = 7) from quiescent (**1**), maturing (type A spermatogonia proliferating; **2**), mature (**3**) and spawning (**4**) fish. **b)** Proposed conversion of androstenedione in cod testis. The main pathway leading to the synthesis of 11-ketotestosterone in cod testis is indicated by black arrows. **A**: androstenedione; **T**: testosterone; **OHA**: 11β-hydroxyandrostenedione; **KA**: 11-ketoandrostenedione; **KT**: 11-ketotestosterone; **OHT**: 11β-hydroxytestosterone. Steroidogenic enzymes indicated are: 11β-hydroxylase (**11βCyp**), 11β-hydroxysteroid dehydrogenase (**11βHsd**), and 17β-hydroxysteroid dehydrogenase (**17βHsd**).

Androgen-dependent Activation of Cod Ar Expressed in Mammalian HEK Cells

The cod *ar* open-reading frame expression vector was co-transfected with an androgen-regulated reporter (MMTV-luciferase) construct, into human embryonic kidney 293-T cells, which are known for having little androgen-metabolizing enzyme activities (Wu *et al*. 1993) and no evidence for an endogenous Ar (de Waal *et al*. 2008). Transfected cells were then incubated

with androgenic (T, KT, 17α-methyltestosterone [MT], 5α-dihydrotestosterone [DHT] and A) and non-androgenic (17β-estradiol [E_2], 17α,20ß-dihydroxy-4-pregnen-3-one [DHP] and cortisol) steroids. The parallel curves produced by the androgens are shown in Figure 2a. The EC_{50} value was averaged for each androgen and the ligand with the highest potency to transactivate cod Ar was MT (EC_{50} = 0.17 ± 0.03 nM), followed by DHT (EC_{50} = 1.45 ± 0.83 nM), T (EC_{50} = 2.01 ± 0.44 nM) and KT (EC_{50} = 13.1 ± 6.9 nM), the least potent androgen being A (EC_{50} = 104.0 ± 61.7 nM). At very high concentrations, DHP (10^{-5} M) and in particular cortisol (10^{-6} and 10^{-5} M) activated the MMTV promoter while E_2 was completely inactive (Fig. 2b). No activation of the receptor was observed when no steroid was added to the medium or when empty plasmid was transfected and cells exposed to MT (Fig. 2b).

Saturation and Ligand-competition Assays

Next to transactivation, we investigated ligand binding to the cod Ar using [^{3}H]-testosterone ([^{3}H]-T) as ligand. Saturation was reached at ~3 nM [^{3}H]-T, and binding remained constant up to a concentration of 50 nM. The saturation curve showed a single population of binding sites and the dissociation constant (K_d) was 1.01 nM (Fig. 2c). When empty vector was used, total binding and non-specific binding counts did not differ and therefore a K_d could not be determined.

Ligand-binding competition assays were performed using 2 nM of [^{3}H]-T as tracer to determine the relative affinities of different natural androgens to cod Ar. The parallel binding curves showed competitive binding between the various non-radioactive steroids and the tracer (Fig. 2d). KT (K_i = 1.9 ± 0.2 nM) was the strongest competitor of tritiated T for binding the cod Ar, followed by A (K_i = 32.7 ± 11.9 nM), OHT (K_i = 66.7 ± 10.6 nM) and KA (97.9 ± 49.2 nM). OHA presented the highest K_i (219.3 ± 10.8 nM).

Expression Profile of cod *ar* mRNA During Larval Development and in Adult Tissues

Using real-time, quantitative PCR we detected high amounts of cod *ar* mRNA maternally deposited into the embryo 24 hours after fertilization. The levels fell rapidly during the first days of development to low levels in larvae of 5 – 6 mm length, when first feeding was about to commence (Fig. 3a). The lowest levels were quantified in larvae of approximately 10 mm, just before the start of morphological sex differentiation of the gonads (unpublished observations). From then on, the *ar* mRNA levels increased steadily with the development of the larvae (samples were up to 57 mm).

Analyzing six different adult organs, no significant difference was found between male

and female cod regarding *ar* transcripts levels in the same tissue type. However, testis tissue showed a significant higher *ar* mRNA level than ovarian tissue. Comparing all organs studied, head kidney presented the lowest values and intestine and liver the highest values, followed by spleen, gill and heart (Fig. 3b).

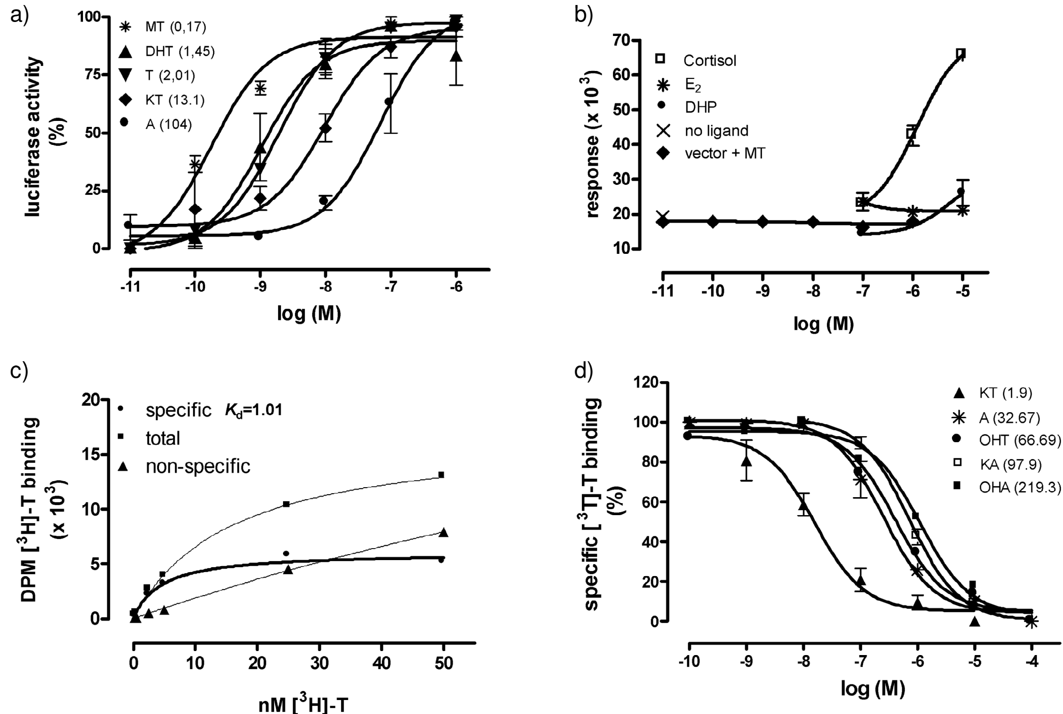

Figure 2. ***In vitro* transactivation of cod Ar**. **a)** Normalized results of ligand-induced activation of cod Ar incubated with increasing concentrations (10 pM to 1 µM) of different androgens; the averaged EC_{50} value of each steroid is given between brackets; each curve represents the mean ± standard errors of three independent assays run in duplicates. **b)** Absolute values of cod Ar activation after incubation with increasing concentrations of different non-androgenic steroids and with medium without ligand; empty vector was also transfected and stimulated with MT (the strongest ligand in activating cod Ar) to test the presence of endogenous androgen receptor in the transfected HEK-293T cells. **c)** Saturation binding curve of cod Ar transfected in HEK-293T cells using [^{3}T]-T; specific binding was determined by subtracting non-specific binding counts from the total binding counts; the graph is representative for three assays run in duplicates. **d)** Normalized competitive binding curves showing relative affinities of natural androgens for the cod Ar; assays were conducted with increasing concentrations (0.1 nM to 0.1 mM) of each steroid in competition with fixed 2 nM [^{3}T]-T; averaged K_i value of each steroid is given between brackets; each curve represents the mean ± standard errors of three independent assays run in duplicates. **T**: testosterone; **KT**: 11-ketotestosterone; **MT**: 17α-methyltestosterone; **DHT**: 5α-dihydrotestosterone; **A**: androstenedione; **OHA**: 11ß-hydroxyandrostenedione; **OHT**: 11ß-hydroxytestosterone; **KA**: 11-ketoandrostenedione; **E$_2$**: 17ß-estradiol; **DHP**: 17*α*,20*β*-dihydroxy-4-pregnen-3-one.

Under normal light (NL) conditions, the gonado-somatic index (GSI) remained at low values (0.2 – 1.0%) from June to September in animals with quiescent testes and during spermatogonial proliferation (Fig. 3c). GSI values increased significantly when meiosis and spermiogenesis was initiated (October), and reached peak levels in fish during full

spermatogenesis (November) and during spawning (February). In fish exposed to constant light (LL), GSI values were low and no significant changes in gonad weight over time were recorded (Fig. 3c). However, this group was heterogeneously composed and contained males with quiescent testis (n = 11) and males showing spermatogenic activity (n = 15) but on a very small scale and with elevated levels of apoptosis (see Almeida *et al*. 2009), so that significant differences with regard to GSI values were apparent between the two subgroups in the LL group (Fig. 3c, inset).

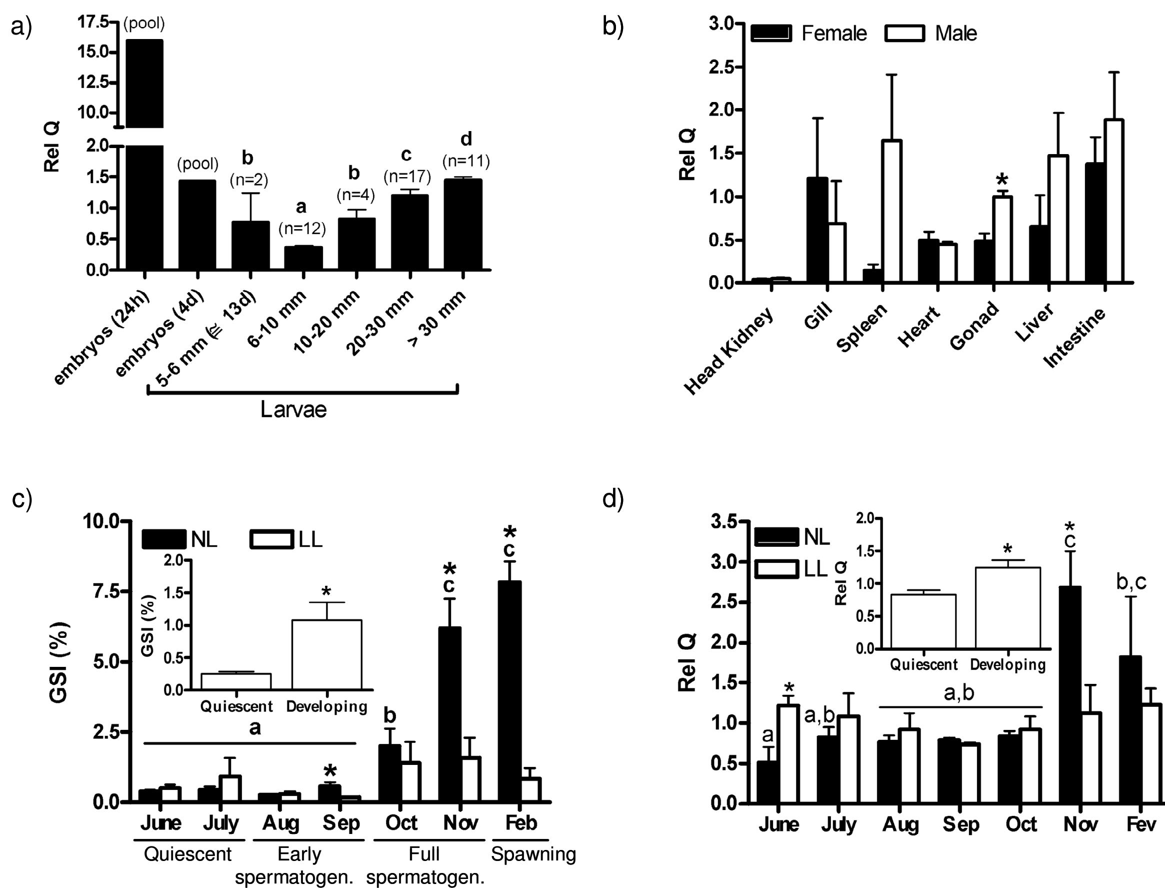

Figure 3. **Expression of cod *ar* mRNA and gonado-somatic index**. **a)** *ar* expression in cod embryos throughout early larval development; average of all quantitative values was used as calibrator for calculating the relative quantities (**Rel Q**). **b)** *ar* expression in adult organs of male and female cod (n = 3); averaged testis quantitative values was used as calibrator for calculating the relative quantities. **c)** Gonado-somatic index (**GSI**) of male cod kept under natural (**NL**) or constant light (**LL**) during one reproductive cycle; inset shows difference in GSI between the two LL sub-groups (quiescent and developing testis); histological classification of the gonads per month (NL) is presented along the x axis. **d)** *ar* expression in testes of cod kept under NL or LL during one reproductive cycle; relative quantities were calibrated with the average of quantitative values from the NL fish; inset shows difference in *ar* expression between the two LL sub-groups. For statistical analysis, only the larvae values were used in **a**, due to number of samples (embryos were pooled) and the values were log transformed in **c** and **d**. In **b**, asterisk indicates significant difference ($P \leq 0.01$); in **c** and **d** columns labeled with the same letter do not differ significantly ($P \leq 0.05$) between months within the NL group, and asterisk show significant difference between NL and LL in the given month.

In the NL group, the expression of testicular *ar* mRNA was low but clearly detectable in quiescent testes found in June and July. The levels did not change significantly when spermatogenesis had started (spermatogonial proliferation), or proceeded to meiosis, spermiogenesis and the start of spermiation in October (Fig. 3d). However, a significant up-regulation of *ar* mRNA levels was recorded when testicular mass had increased to nearly maximal levels (GSI 7.8% ± 0.7) with spermatogenesis being in full swing in November, after which a small decrease was recorded in fish in spawning condition in February (Fig. 3c and d). In cod kept under LL, the *ar* expression varied somewhat but did not show statistically significant differences over time. Sorting this group according to the absence or presence of spermatogenic activity revealed that –similar to difference in GSI values– *ar* mRNA levels were significantly higher in those males showing spermatogenic activity (Fig. 3d, inset). A significant difference in testicular *ar* mRNA levels between photoperiod treatment groups was observed in two months: in June, when the sample in the LL group was composed of four animals that all showed spermatogenic activity while testes were quiescent in the NL control group, and in November when a strong up-regulation was observed in the NL group (Fig. 3d).

DISCUSSION

Among a broad range of different physiological roles, androgens are critical regulators of spermatogenesis throughout vertebrates. In fish, androgens stimulate in particular the differentiation of type B spermatogonia and their rapid proliferation (Miura *et al*. 1991; Miura *et al*. 2002; Cavaco *et al*. 1998).

The present study aimed at investigating reproductive aspects of the androgen signaling system in Atlantic cod. We cloned a cod *ar* open-reading frame that predicted an androgen receptor protein of 718 amino acids with a calculated molecular weight of 79,53 kDa. It is well-known that androgen receptors, as other nuclear receptors, are modular proteins composed of a variable transactivation domain (TAD) at the N-terminus, a highly conserved DNA binding domain (DBD), a hinge region, and a conserved ligand binding domain (LBD) at the C-terminus (Evans, 1988; Beato, 1989). In the cod Ar, these conserved domains could also be identified. Moreover, in the DBD, the position of the 10 cysteine residues, of which eight constitute the two zinc fingers motifs, and the P box were also conserved, which are very important for recognizing target gene *cis* elements. In the D box (AGRND) of the cod Ar, the glycine (commonly present in mammalian ARs), was substituted by a serine (ASRND), as observed in Ar of other fish as well. Moreover, a putative leucine zipper motif was found in the C-terminal

region of the cod Ar; this structure is well conserved in nuclear receptors and required for their dimerization and transcriptional regulation (Pfahl, 1993).

Several studies have used mammalian cell lines to examine transactivation function of fish Ars (Takeo and Yamashita, 1999; Todo *et al.* 1999; Ikeuchi *et al*. 1999; Touhata *et al*. 1999; Wilson *et al*. 2004; de Waal *et al*. 2008). In the present study, HEK 293-T cells were co-transfected with the cod *ar* expression vector and with a reporter construct under the control of an androgen-responsive (murine mammary tumor virus) promoter and then incubated with different ligands. All androgens tested stimulated the androgen-dependent expression of the luciferase gene through the cod *ar* in a dose-dependent manner. When cells were transfected only with MMTV-Luc plasmid, androgens were unable to induce luciferase expression, indicating that there was no interference of an endogenous Ar. Exposure to estradiol or progesterone did not induce reporter gene expression, confirming the androgen specificity of the receptor. However, high concentrations (1 or 10 µM) of cortisol did. This cross activation phenomenon is considered physiologically not relevant since cortisol plasma levels in cod remain well below 1 µM concentrations (Perez-Casanova *et al*. 2008). The synthetic androgen methyltestosterone was the best ligand as regards cod Ar transcriptional activity, followed by dihydrotestosterone, a potent androgen in mammals but usually not present in fish (Borg, 1994). Among the endogenous androgens, testosterone (T) potently transactivated the cod Ar, followed by the ~5-fold less potent 11-ketotestosterone (KT), and the ~50-fold less potent androstenedione (A). Regarding binding to Ar, T presented a dissociation constant of 1 nM. Similar results were reported for the zebrafish Ar activation by androgens (de Waal *et al*. 2008): T and KT showed similar activities, while 15-50-fold higher levels of A were required for Ar binding and activation. Other 11-oxygenated androgens were still 2- to 7-fold less active than A.

Previous work analyzing cod T and KT plasma levels in a large number of individual samples (Almeida *et al*. 2009) showed that the levels of KT were usually higher than those of T and that peak levels of both androgens were observed during spawning, similarly to the findings of the present study. The different sampling regime of the previous study allowed, however, detecting an increase in plasma T, but not KT, levels already during the start of full spermatogenesis in October. In general, the T and KT concentrations up until full spermatogenesis were 0.5-2 ng/ml, equivalent to ~2-8 nM, a concentration well suited to activate the Ar in cod. With a higher potency than KT and an earlier increase in plasma levels, it seems that T is the most important androgen during the early stages of cod male maturation. The situation changes towards spawning. All plasma androgens except 11ß-hydroxytestosterone (OHT) increased. Interestingly, this was particularly prominent for 11-ketoandrostenedione (KA) and androstenedione (A), two androgens that are less active as Ar

agonists. It is reasonable to assume that an increased 17α-hydroxylase (Cyp17α) activity is required for the elevated androgen production in spawning fish. However, compared to A and KA, relatively small amounts of T and KT were found, suggesting that the 17β-hydroxysteroid dehydrogenase (17βHsd) mediated production of biologically active 17β-hydroxylated steroids may be restricted in the testis to avoid the androgen-mediated down regulation of Cyp17α activity that was reported in fish (Cavaco *et al*. 1999) and mammals (Payne and Youngblood, 1995). Since several non-gonadal tissues (Schulz and Blüm, 1991), including red blood cells (Mayer *et al*. 1990) show 17βHsd activity, we hypothesize that the testis produces biologically less active androgens (A and KA in the case of cod) to protect its steroidogenic activity from down regulation. These androgens then serve as precursors and are converted to T and KT at extra-testicular sites. In the case of A, however, the levels found in blood plasma in spawning fish surpassed 50 nM, which potentially is high enough to activate the cod Ar.

The *ar* mRNA levels decreased during early development in Atlantic cod embryos, probably reflecting the consumption/decay of maternally contributed *ar* mRNA. Similar quantitative changes have been described in zebrafish (Hossain *et al.* 2008) and studies on the localization of *ar* expression in zebrafish embryos and larvae suggested that this nuclear receptor may play yet ill-defined roles during early stages of ontogenesis in different tissues, such as brain and kidney (Gorelick *et al*. 2008).

Larval cod *ar* transcript levels started to re-increase when approaching the size range in which gonadal sex differentiation can be observed morphologically (20-30 mm; unpublished information). For testis morphogenesis in mammals, AR protein expression is indispensable (Werner *et al*. 1996). Also early differentiating ovaries seem to express functional Ar in cod, considering that androgen treatment of cod larvae starting just prior to morphological sex differentiation can induce female-to-male sex change (Haugen T, IMR, Norway; unpublished information). In adult cod, *ar* mRNA was found in all seven tissues analyzed from both sexes, with no significant difference between sexes, except for gonadal tissue (i.e. *ar* overexpression in males).

Under normal light conditions (NL), testicular cod *ar* mRNA levels did not change significantly during the transition from quiescence to spermatogonial proliferation or when meiosis or spermiogenesis was initiated. However, after having entered the rapid growth phase, an up-regulation of *ar* mRNA levels was recorded. The testicular weight gain mainly reflects an increase in germ cell number that is fueled by the consecutive rounds of mitotic proliferation of spermatogonia, resulting in a geometric increase in the number of spermatogonia (Almeida *et al*. 2008). Previous work showed that cod *ar* mRNA was predominantly located in Sertoli cells in contact with these rapidly proliferating spermatogonia

(Almeida *et al*. 2009). Therefore, we assume that the increase in *ar* mRNA levels recorded from October to November reflects an increase in the testis of the population of Sertoli cells that are in contact with rapidly proliferating type B spermatogonia. In context with the above discussed androgen levels, it seems possible that the start of this rapid growth phase in October is initiated by T, considering that the plasma T levels start to increase significantly in this month in the NL group (Almeida *et al*. 2009).

Interestingly, the subgroup of LL-exposed males that did show spermatogenic activity also showed significantly elevated *ar* mRNA levels and GSI, though GSI values were much lower than in developing NL-exposed fish. While also the plasma T (but not KT) levels were significantly higher in the fish showing some spermatogenic activity in the LL group (1.4 compared with 0.7 from quiescent males; Almeida *et al*. 2009), their T levels were still lower than those found in NL fish showing spermatogenic activity (Almeida *et al*. 2009). The combination of slightly elevated T levels and increased *ar* mRNA expression might explain the observation that these males did show some spermatogenic activity despite the inhibitory photoperiod condition, while their relatively low androgen levels (in relation to the NL group) might explain the observed elevated level of apoptosis (Almeida *et al*. 2009).

The mean *ar* mRNA level was still relatively high in spawning fish from the NL group, although Sertoli cells in contact with rapidly proliferating spermatogonia were no longer present in February, so that the functional context discussed for the increased expression levels in fish during full spermatogenesis is no longer relevant in spawning fish. However, Sertoli cells remain in the fully mature testis as an epithelial lining of the spermatogenic tubules and are required later on in post-spawning fish to remove the residual spermatozoa (Almeida *et al*. 2008). Unfortunately, detection of *ar* mRNA by in situ hybridization required the use of cryosections (Almeida *et al*. 2009) and we did not have access to fresh tissue of spawning or post-spawning males yet to investigate if *ar* mRNA expression was detectable in Sertoli cells at these advanced stages of development.

The steroidogenic system may be an additional functional context for the Ar during spawning, considering that mammalian Leydig cells are Ar-positive and show Ar-mediated regulation of steroidogenic genes expression (Payne and Youngblood, 1995), and that androgen production in fish can be modulated by androgens (Schulz *et al*. 2008). A strong activation of the steroidogenic system in spawning fish is evident from the increases in circulating levels androgens quantified in this and in a previous study (Almeida *et al*. 2009). If this is also associated with effects on Leydig cell *ar* mRNA expression, remains to be investigated.

In summary, we cloned the cod androgen receptor, characterized its pharmacology, analyzed its binding and activation characteristics using different androgenic and non-

androgenic steroids, studied its expression during and after larval development, and in testis tissue developed under normal and experimental (constant light) conditions, and studied a range of plasma androgens. From our results, we propose that first T and then T and KT are likely to be the physiological ligands for the cod Ar. Its expression pattern suggested roles for the Ar during early stages of ontogenesis, during sex differentiation, and in the developing testis of Atlantic cod.

ACKNOWLEDGMENT

The authors gratefully acknowledge Dr. Trine Haugen and Ms Sissel Waage Kalvenes, Austevoll Research Station, Norway, for providing the larvae cDNAs used in this study, and for technical assistance, respectively.

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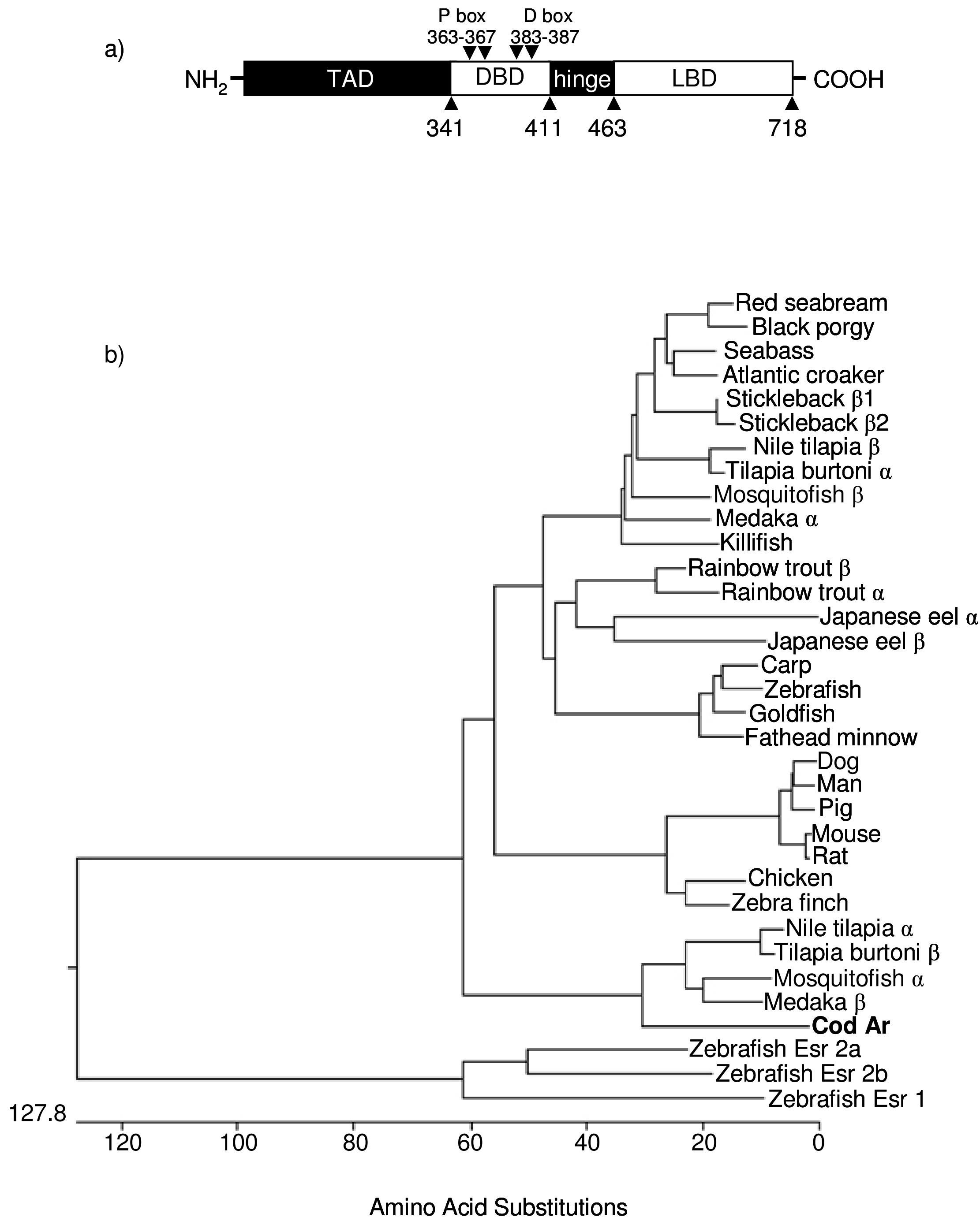
Supplemental Table 1

Primer name	Sequence
2197[a]	5′-GAAGAGVAGGARGGCCTTCATGCAGAGRAA-3′
2198[a]	5′-CTCACHTGTGGMAGCTGCAAGGTKTTCTTCAA-3′
2230	5′-GCAAAGTACAGCATGGAGCCATTAGTGAGCGTGTAAG-3′
2231	5′-TCTTCTACCCTGCTTCGCGTTTGAGTGGC-3′
2237	5′-CCACAAGCTACGCAGCCTGCTCCCAGA-3′
2238	5′-CTGCAGGCCATTGAACCCATGGTGGTCAATG-3′
2269[b]	5′-AGCCACCATGACCGAGTCTTCGTATGGTAAGCCAGTCCCCTC-3′
2270	5′-GGGTCCTAGCCTGCCTTATGGAAAAGAATGGGCTTG-3′
2655	5′-ACTTTGCTCCAGACCTGATCTTTG-3′
2656	5′-GAGTTGCATCCTCACACAGTGTTC-3′

[a]Primers 2197 and 2198 are degenerative primers, in which H = A, T or C, K = G or T, M = A or C, R = G or A, and V = G, A or C. [b]Primer 2269 contains a Kozak consensus sequence (underlined; Kozak, 1984)

Supplemental Figure 1



Cod Ar protein. **a)** Schematic representation of cod Ar structure and predicted domains: transactivation domain (**TAD**), DNA-binding domain (**DBD**) with P and D boxes, hinge region and ligand-binding domain (**LBD**). **b)** Phylogenetic analysis of selected vertebrates and cod Ar proteins; amino acid sequences were obtained from GenBank databases; phylogenetic tree was constructed using Neighbor-Joining method; zebrafish estrogen receptors were used as out group, to root the tree. Accession numbers: *Homo sapiens* AR (M23263); *Mus musculus* AR (AL844866); *Rattus norvegicus* AR (M20133); *Sus scrofa* AR (AF202775); *Canis lupus* AR (AF197950); *Gallus gallus* AR (AB193190); *Taeniopygia guttata* AR (BK005685); *Oreochromis niloticus* Arα (AB045211) and Arβ (AB045212); *Chrysophrys major* Ar (AB017158); *Anguilla japonica* Arα (AB023960) and Arβ (AB025361); *Gasterosteus aculeatus* Arβ1 (AY247206) and Arβ2 (AY247207); *Carassius auratus* Ar (AY090897); *Oncorhynchus mykiss* Arα (AB012095) and Arβ (AB012096);

Oryzias latipes Arα (AB076399) and Arβ (EU100398); *Acanthopagrus schlegelii* Ar (AY219702); *Dicentrarchus labrax* Ar (AY647256); *Micropogonias undulatus* Ar (AY701761); *Astatotilapia burtoni* Arα (AF121257) and Arβ (AY082342); *Gambusia affinis* Arα (AB174849) and Arβ (AB182329); *Kryptolebias marmoratus* Ar (DQ339105); *Spinibarbus denticulatus* Ar (EU517118); *Danio rerio* Ar (EF153102); *Pimephales promelas* Ar (AY727529); *Danio rerio* Esr1 (AY307098), Esr2a (AF349412) and Esr2b (AF349413).

6

Summarizing Discussion

SUMMARIZING DISCUSSION

Male Atlantic Cod Puberty

In vertebrates, puberty is, by definition, the process by which a sexually immature individual acquires, for the first time, the capacity to reproduce (Okuzawa 2002). In juvenile males this process is characterized by the onset of spermatogenesis (Schulz and Miura 2002). Despite the importance of Atlantic cod for the economy of the Northern countries and the problems related to precocious puberty in cod aquaculture, the first testis maturation, including the context of the endocrine signalling between pituitary and testis, had not been investigated in detail yet. Therefore, in the present thesis, efforts were directed to study the roles of important components of the regulatory system governing spermatogenesis in pubertal cod. We examined the expression of pituitary gonadotropins, of testicular gonadotropin and androgen receptors, as well as the plasma levels of different androgens, in close context with morpho-physiological aspects of the spermatogenic process in this gadoid. On the basis of previous research, photoperiod treatment regimes were applied to generate experimental groups with largely differing stages of testis development, expanding our research material for understanding the photoperiod-modulation of the complex process of Atlantic cod male puberty.

The Atlantic cod is a seasonal spawner undergoing annual reproductive cycles in response to environmental cues, in particular photoperiod. Seasonal reproduction is synchronized with environmental signalling to ensure that hatching and larval development occurs under favourable conditions. In cod, as soon as day-length starts to decrease after the summer solstice, the photoperiod information relevant for puberty is perceived by the brain, probably via the lateral eyes and/or the pineal gland (Fálcon *et al.* 2007), and triggers profound changes in the neuroendocrine system that controls the release of the neuropeptide gonadotropin-releasing hormone (Gnrh) by hypothalamic neuroendocrine neurons. In several teleost species, two or three different forms of both Gnhr ligands and Gnrh receptors were found (Kah *et al.* 2007). Respective information still lacks for Atlantic cod, but we assume that – similar to other fish (Dubois *et al.* 2000) – one of the Gnrh-producing cell populations is situated in the ventral hypothalamus and directly innervates the pituitary with axons ending in the vicinity of the gonadotrophs.

In certain seasonally breeding mammals, the pineal hormone melatonin acts as a neuroendocrine mediator in the perception of photoperiod signalling and modulates, according to daylenght changes, the activity of KiSS-1 neurones in the release of the neuropeptide

kisspeptin (KiSS-1; Revel *et al.* 2007). The role of melatonin in fish reproductive activity is not clarified yet, and melatonin administration can have pro- or anti-gonadal effects in different species (Joy and Agha 1991; Randal *et al.* 1995; Bromage *et al.* 1995; Mayer *et al.* 1997a,b; Taranger *et al.* 2004). However, in other mammals where melatonin is not directly involved in the photoperiodic signalling or where other environmental or internal signals are relevant, the activity of the Kiss1 neurons can be regulated by steroid hormones (Gottsch *et al.* 2006), or by metabolic signals like leptin (Tena-Sempere 2006). In any case, the modulation of Kiss1 release by factor(s) reflecting information of relevance for the timing of reproduction of a given species is a decisive maneuver in the brain-pituitary-gonad (BPG) axis function. After release, KiSS-1 binds and activates its specific receptor GPR54 in hypothalamic GnRH-neurons, which stimulates the release of GnRH, thereby triggering the onset of puberty in mammals. This results in stimulation of synthesis and secretion of the two pituitary gonadotropins that control maturation of the major gonadal functions, sex steroid synthesis and gamete production and release (Revel *et al.* 2007; Shahab *et al.* 2005; Smith *et al.* 2006). The Kiss-1 peptin/Gpr54 system also has been found in fish and has been implicated in pubertal maturation (van Aerle *et al.* 2008; Filby *et al.* 2008; Mohamed *et al.* 2007; Nocillado *et al.* 2007); two *kiss* genes were identified in zebrafish, medaka, sea bass and goldfish (Kitahashi *et al.* 2009; Felip *et al.* 2008; Li *et al.* 2009) and two *gpr54*s in zebrafish (Biran *et al.* 2008) and goldfish (Li *et al.* 2009). The Kiss/Gpr54 system seems a good candidate for conveying photoperiod signals to the Gnrh system also in Atlantic cod, but has not been the subject of the present thesis.

At the onset of puberty in male cod, the synthesis of Fsh by the pituitary is strongly up-regulated. We speculate that the decreasing photoperiod after the summer solstice triggers, possibly via Kiss1/Gpr54, an increased Gnrh release that, via a yet unknown mechanism, specifically stimulates pituitary *fshb* gene expression, and possibly also Fsh release. This would result in changes in testis morphology and physiology, constituting pubertal testis activation, via binding and activation of the Fsh receptor (Fshr) in the immature testis (Chapter 4). The immature or quiescent testis contains, in each of its several lobes, type A spermatogonia as the only germ cell type, which compose the germinal epithelium in close association with Sertoli cells; a lumen had not formed yet in the testicular parenchyma (Chapter 2). Developmental processes observed at the commencement of puberty, such as the formation of new cysts with spermatogonia, and the transition of small cysts with slowly proliferating type A spermatogonia to large cysts with rapidly proliferating type B spermatogonia, may reflect Fsh-mediated activation of Sertoli cells. This recruitment and subsequent growth of the cysts, involving also mitosis of the cyst-forming and germ-cell supporting Sertoli cells (Schulz *et al.* 2005; Leal *et al.* 2009), does not take place randomly in the cod testis parenchyma. Although

small cysts containing a single type A spermatogonium can be found throughout a testis lobe, the formation of new cysts occurs preferentially in the distal and peripheral areas, constituting a germinative zone. This zone is responsible for the appositional growth of each lobe and for the gradient of maturation that is noted when germ cells progress through spermatogenesis (Chapter 2). The presence of this germinative zone might reflect the existence of a specific microenvironment favouring spermatogonial proliferation and differentiation, and possibly is the target of regulatory processes. After all, spermatogonial proliferation in cod (under NL conditions) can be noticed during a specific period, from August to the beginning of January. Our data on the expression profile of *fshb* would be compatible with a role for Fsh in the regulation of spermatogonial proliferation.

Germ cell loss, although relatively rare in cod spermatogenesis, occurs mainly during this proliferative phase. Apoptosis in proliferating spermatogonia, on the other hand, might be suppressed by androgens via the Sertoli cell, since the androgen receptor (*ar*) mRNA is preferentially localized in Sertoli cells contacting mitotic clones of spermatogonia, and since low steroid levels are associated with high incidence of germ cell loss during the spermatogonial phase (Chapters 2 and 3).

Spermatogenesis within a cod population is not a synchronized process, neither in wild (Burton *et al.* 1997) nor in captive fish (Chapter 2). However, meiosis and spermiogenesis generally start in October in males reared in Austevoll, Norway (60^0N). A slow increase in T plasma levels is noted from October (Chapter 3). This situation is similar to findings in other fish species, in which plasma androgen levels stay low or show only gradual increases during a period of considerable testis growth and elevated Fsh plasma levels or pituitary expression (Kusakabe *et al.* 2006; Gomez *et al.* 1999). Nevertheless, steroid release can be stimulated directly by Fsh (Planas *et al.* 1993; Planas and Swanson 1995; Zmora *et al.* 2007), probably via Fshr expression by Leydig cells (Ohta *et al.* 2007; García-López *et al.* 2009). While we have no data on the presence of Fshr on Leydig cell in cod testis, it seems possible that the slight but steady rise in plasma T levels, in particular from October to December, might reflect steroidogenic activity of Fsh (Chapters 3 and 4). Taken together, Fsh and androgens, in particular T, seem important signalling molecules at the beginning of spermatogenesis, via stimulatory and protective paths.

One of the androgen effects in eel testis is the release of the TGFβ family member activin by the Sertoli cell for stimulating spermatogonial proliferation (Miura and Miura 2001). Another TGFβ factor, anti-Müllerian hormone, which prevents differentiation of spermatogonial stem cells and hence the start of spermatogenesis (Miura *et al.* 2002) is down-regulated by androgens. Interestingly, insulin-like growth factor 1 (Igf1) is required as a permissive factor for the

androgen-stimulatory effects in eels (Miura and Miura 2001). It would be interesting to examine if Igf1 is produced by Sertoli cells, perhaps in an Fsh-dependent manner.

Concurrent with the conclusion of meiosis and spermiogenesis, the GSI values attain their highest values from January to March, approaching more than 8% of total body weight (Chapter 2). As spermatogenesis proceeds, cysts reach the spermiation stage and the tubule lumen become filled with free spermatozoa in a maturational wave from the collecting duct zone towards the lobe's periphery, characterizing spawning conditions (Chapter 2). Towards, during, and at the end of the spawning season, T and KT plasma levels and *lhb* expression (accompanied one month later by testicular *lhr* expression) jointly increase, stay high, and then decrease parallel to each other, so that they are possibly integrated in a positive feed back loop (Chapters 3 and 4).

A very interesting feature observed in the steroidogenic activity of cod testis during the spawning season is the auto-protective manner in which androgens are produced. Weak Ar agonists, such as 11-ketoandrostenedione and androstenedione, are present in the blood in high concentrations. If this reflects high testicular production, it may be a mechanism avoiding the androgen-mediated down-regulation of Cyp17a, which would, otherwise, limit the androgen production capacity (Cavaco *et al.* 1999; Payne and Youngblood 1995). On the other hand, the physiologically necessary high concentrations of T and KT during spawning - probably in the context of reproductive behaviour and for developing secondary sexual characteristics - can be achieved by extra-gonadal 17β-hydroxysteroid dehydrogenase activity (Schulz and Blüm 1991; Mayer *et al.* 1990).

With the completion of spawning, pituitary *lhb* and *fshb*, testicular *lhr* expression, and androgen plasma levels, are all down regulated, perhaps due to the photoperiod signal of increasing day-length towards summer and both, gamete and steroid production revert to minimum activity levels, with little change until late summer, when shortening days are triggering the next cycle of maturation.

Effects of Light on Atlantic Cod Testis Pubertal Maturation

Cod reproduction is more sensitive to changes in photoperiod than to changes in temperature (Cyr *et al.* 1998). Therefore, the effects of constant light (LL) applied in outdoor, light-proof tanks and starting at different points of pubertal maturation were analyzed as regards changes in the endocrine signaling towards the gonad and consequent alterations in testis functions, namely androgen and gamete production.

While previous studies had already made use of different light regimes to modulate cod puberty (Hansen *et al.* 2001; Norberg *et al.* 2004; Skjæraasen *et al.* 2004; Taranger *et al.* 2006;

Karlsen *et al.* 2006; Davie *et al.* 2007), data regarding the light effects on the signaling between pituitary and testis were still missing. Nevertheless, these data were important for elucidating critical aspects of the photoperiodic-modulation of Atlantic cod first testis maturation.

The general response to LL is "silencing" testis development, although the type of response also depends on the stage of development in which fish were at the start of the LL treatment. The pituitary gonadotropin subunits, for instance, can be either down or up-regulated by LL. If constant light is applied at mid-winter, the final steps of testis development, androgen plasma levels, pituitary expression of *lhb* and testicular expression of its receptor, all are strongly stimulated, then reaching higher values than in the NL group. The production of new spermatogenic cysts is immediately terminated and germ cells finish their development, leading to a precocious spawning activity, sperm release and entry into the post-spawning/spent condition (Chapters 3 and 4).

On the other hand, extending the long photoperiod typically seen in summer seems incompatible with activating the BPG axis. Gonadotropin subunit and testicular *lhr* expression and androgen production remain inactive. Moreover, spermatogenesis is either fully blocked (in about 65% of this population; Chapter 3) or takes place in a quantitatively and qualitatively restricted manner: only a limited number of cysts is recruited into spermatogenesis, the maturational gradient in the testis lobes is not established, and the incidence of germ cell loss is very high. It appears that significant deviation from an optimal endocrine environment is not compatible with quantitatively normal spermatogenesis.

While also the plasma T (but not KT) levels are slightly higher in the fish showing some spermatogenic activity in the LL group, their T levels are still lower than in NL fish showing spermatogenic activity. The combination of slightly elevated T levels and testicular *ar* mRNA expression might explain the observation that these males did show some spermatogenic activity despite the inhibitory photoperiod condition, while the comparatively (in relation to the NL group) low androgen levels might be the reason for the elevated incidence of apoptosis (Chapters 3 and 5). A prominent difference between LL and NL fish is that during the period of first slowly and then rapidly increasing GSI in NL fish, a clearly elevated pituitary expression of *fshb* mRNA is observed in the NL but not in the LL group. Hence, the absence of high *fshb* expression might be responsible for the low number of cysts being recruited into maturation in the LL-exposed animals (Chapters 3 and 4).

These observations lead us to assume that beside an individual's responsiveness of the brain-pituitary-gonad system to the LL challenge, also each cyst may be an independent functional unit that does or does not embark onto spermatogenesis in response to a given endocrine signaling.

This thesis describes the pubertal testis maturation in Atlantic cod, combining morphological and endocrine features, such as expression of pituitary gonadotropins, testicular gonadotropin and androgen receptors and androgen plasma levels, and discusses possible interactions between these signals to jointly regulate pubertal testis development. While several interesting findings were made that merit further research, mechanisms upstream of the pituitary testis axis will possibly trigger events on the pituitary and gonadal levels. For further characterization of the mechanism by which the photoperiod information is processed in the brain, the cod appears to be a well-suited model, given its exquisite sensitivity to photoperiod manipulation. For instance, though some work on cod melatonin and its regulation by light has been done (e.g. Migaud *et al.* 2007), a proper link between the pineal gland and reproduction has not been established. Moreover, revealing the presence, localization and activity of the Kiss1/Gpr54 system in the regulation of Gnhr/Gnrhr-mediated stimulation of synthesis and release of gonadotropins will, on the one hand, serve as the basis for applied research directed towards improving the sustainability of cod aquaculture, and on the other hand open the access to revealing the input towards the Kiss1-producing neurons, a question that has remained unanswered in all vertebrates so far.

The present thesis provides information on male cod puberty responding to both academic and applied interests. New insights were gained into the morphological organization of cystic spermatogenesis and, regarding the endocrine mechanisms regulating puberty in fish, establishing Atlantic cod as an interesting model for future research work. Moreover, from the more applied point of view, the data presented here show that while constant light is rather effective in preventing testis growth and has negative effects of low plasma androgen levels, spermatogenesis still takes place to a limited degree in ~35% of the population, so that the possibility of "genetic contamination" of remaining wild stocks is not fully contained by photoperiod regimes. This suggests that other possibilities to deal with these items should be evaluated as well, such as monosex populations.

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SUMMARY

Puberty is the process by which a juvenile acquires for the first time the capacity to reproduce sexually, a critical basis for the conservation of a species. In general, the biological processes constituting the first gonadal maturation and the mechanisms involved in this process are similar among vertebrates. Internal and/or external signals trigger the secretion of gonadotropin-releasing hormone (Gnrh) by neurosecretory neurons in the ventral forebrain, and Gnrh stimulates the synthesis and secretion of two pituitary hormones, the gonadotropins known as follicle-stimulating hormone (Fsh) and luteinizing hormone (Lh). These two glycoproteins are the major hormones regulating the remarkable structural and functional changes occurring in the pubertal gonads during the activation of the two principal gonad tasks, the production of gametes and sex steroids.

In context with the male-biased problems of precocious testis maturation in farmed cod, the present thesis focuses on male puberty. Pituitary *fsh* gene expression starts to increase at the beginning of testis development (proliferation of spermatogonia), followed by a slow elevation in testosterone plasma levels. With rapidly continuing spermatogenesis, which takes place in a peculiar spatio-temporal organization in the testicular lobes, high gonado-somatic indexes of up to 16 % are attained. Close to spawning, testosterone and 11-ketotestosterone, two potent ligands for the cod androgen receptor (Ar), peak in parallel with pituitary *lh* and testicular *lhr* (Lh receptor) gene expression, while *fshr* seems to be expressed in a constitutively manner in the cod testis. It is possible that attaining high plasma levels of potent androgens involves an auto-protective manner of androgen production, where weak Ar ligands, such as androstenedione and 11-ketoandrostenedione, are produced by the testis, thereby avoiding androgen-induced down-regulation of steroidogenic enzymes, while conversion to 17ß-hydroxylated, potent androgens would occur in extra-gonadal tissues. After spawning, pituitary gonadotropin expression and androgens plasma levels decrease to basal levels, and only non-proliferating type A spermatogonia are present as germ cell population in the testis, conditions characterizing the spent condition.

Like in many other teleost fish living at high latitudes, seasonally changing parameters are crucial environmental cues for cod. In this context, the present thesis also provides important information on the effects of constant light, a commonly used tool to prevent gonad maturation in cod farms, on the activity of the pituitary-testis axis. For instance, we found that even under constant light conditions, some males can escape the light-mediated inhibition and develop, associated with elevated pituitary *lh* and testicular *ar* expressions, spermatogenic

activity including sperm production, albeit in a qualitatively and quantitatively compromised manner.

SAMENVATTING

Puberteit is het ontwikkelingsstadium, waarin een individu voor het eerst de capaciteit verwerft om zich voort te planten, de noodzakelijke basis voor de instandhouding van de soort. In het algemeen verloopt dit ontwikkelingsproces en de daarbij betrokken mechanismen bij verschillende gewervelde diersoorten op een vergelijkbare manier. Interne en/of externe invloeden brengen de secretie van gonadotropin-releasing hormoon (Gnrh) door neurosecretoire neuronen in de ventrale voorhersenen op gang. Vervolgens stimuleert Gnrh de synthese en secretie van twee hormonen in de hypofyse, de gonadotropines follikel-stimulerend hormoon (Fsh) en luteïniserend hormoon (Lh). Deze twee glycoproteïnen zijn de meest belangrijke hormonen, die de opmerkelijke stucturele en functionele veranderingen in de puberale gonaden reguleren tijdens de activatie van de twee primaire taken van de gonade, nl. de productie van gameten en geslachtssteroïden.

Problemen die resulteren uit een vervroegd optredende puberteit doen zich in de aquacultuur, ook bij de kabeljauw, vooral bij mannetjes voor. Dit proefschrift is derhalve gericht op de mannelijke puberteit. De hypofysaire *fshb* genexpressie begint toe te nemen bij aanvang van de ontwikkeling van de testis (de proliferatie van spermatogoniën). Dit wordt gevolgd door een geleidelijke stijging van de testosteron spiegels in het bloed. Tijdens de snelle voortgang van de spermatogenese, die trouwens op een bijzondere, niet eerder beschreven, manier in de tijd en ruimtelijk georganiseerd in de testislobben afloopt, worden hoge gonadosomatische indices bereikt van wel 16%. Vlak voor de paaitijd, bereiken testosteron en 11-ketotestosteron, twee potente liganden voor de kabeljauw androgeen receptor (Ar), maximale waarden samenvallend met hoge waardes in hypofysaire *lhb* en testiculaire Lh receptor (*lhr)* genexpressie. De genexpressie van de Fsh receptor (*fshr*) daarentegen verandert minder sterk en lijkt een duidelijke constitutieve component te bezitten. Het zou kunnen dat het bereiken van hoge plasmaspiegels voor potente androgenen alleen mogelijk is via een zichzelfbeschermende manier van androgeen productie in de testis; de Leydigcellen produceren slechts relatief zwakke androgenen, zoals androsteendion en 11-ketoandrosteendion, waardoor een Ar-gemediëerde, afname van de androgeenproductie wordt voorkomen, terwijl de conversie naar de potente, 17β-gehydroxyleerde androgenen in weefsels buiten de gonaden plaatsvindt. Na de

paaitijd dalen de genexpressie van de hypofysaire gonadotropines, de androgeen plasmaspiegels, en de testiculaire *lhr* weer naar het begin niveau, en bestaat de kiemcelpopulatie in de testis alleen uit niet-prolifererende type A spermatogoniën.

Net als voor vele andere beenvissoorten met een habitat duidelijk verwijderd van de evenaar, bevatten veranderende seizoensomstandigheden ook voor de kabeljauw de cruciale milieusignalen. In dit kader beschrijft dit proefschrift ook belangrijke informatie over het effect van continu licht, een veelgebruikt middel in de commerciële aquacultuur om gonadenrijping te remmen, op de activiteiten van hypofyse en testis. Bijvoorbeeld vonden we dat sommige mannetjes kunnen ontsnapten aan deze inhibitie door continu licht en (een kwalitatief en kwantitatief gebrekkige) spermatogenese activiteit kunnen ontplooien, die gepaard gaat met enigzins verhoogde hypofysaire *lhb* en testiculaire *ar* genexpressie.

ACKNOWLEDGEMENTS

From my first e-mail received from Utrecht (from "Dr. Schulz"), containing the formal invitation for a PhD position, until the very last sentence of this thesis, many people, from different places, were at my side, supporting me and helping me in different ways and extents. Each one of them deserve my respect and gratitude.

Firstly, I will be always grateful to Prof. Dr. Luiz Renato de França, who indicated me to the unique opportunity of doing this PhD. Luiz, muito obrigado pela confiança. Essa foi realmente uma oportunidade pela qual sempre lhe serei grata.

My supervisor, far from any doubt, did more than simply supervising my work. He was a real professor in teaching me what I needed for the development of this project, from "what fjords are" to details about cystic spermatogenesis, which I had never worked before. Rüdiger (Dr. Schulz, during my first year), I will be always grateful for your secure guidance, availability and also for your patience (in particular with my writting). Thank you for being so open to discussions and to exchanges of ideas. I hope I can always make use of your vast knowledge about the regulation of spermatogenesis.

I am very glad for having worked with Geir Lasse and Eva Andersson in Bergen. The planings and discussion of results were always so exciting and inspiring that hours would pass as minutes for me. Geir Lasse, thanks for all the opportunities I had every time I wanted to develop something in the cold and either too dark or too light (unbelievable!!!) country. I must say that working with cod in Austevoll was a immense pleasure for me, due to the beauty of the place, the kindness of the staff (whose names I am still not able to pronounce) and, of course, the taste of cod. Evaaaaaa, thanks for introducing me to the molecular biology world, and for your always cheerful company.

Prof. de Rooij, I really appreciate your way to do science, even when you tell me that nights are "not only for sleeping", i.e. Go and Write! It was a great pleasure to have worked in your group. I am grateful for your reading and comments of the manuscripts.

Prof. Adri Thomas, I think you were born to be an excellent mentor. You genuinely concerns about the student's welfare and scientific production. Thank you for the talks, teas and for making me think and realize situations that we normally do not have experie about. You were very important during my PhD.

Ronald and Fritz, I very much appreciate the help with the microscopes. And Miriam, thanks for all the help with the complicated (to me) thousand papers.

From the Endocrinology group, all people I met deserve my acknowledgment, because even in different intensities and periods, each one of them helped me in some way. To Vincent,

Jan Willem, all the portuguese students, Madhavi, Tim, Caaj, Anita, Canan, Pedro, Wouter, Ans, Hermien, Marcelo Emad and Raffa I want to show my gratitude for the work together and/or the happy moments in the lab. I would like to thank in special to Henk, Wytske and Joke for the joyful moments and learnings in the histology lab, cell culture lab and radioactive lab; you three are very special people to me. And to the "Metabolism" people, my thanks for the happy time we worked in the same wing. Prof. Dr. D. J. van der Horst, thank you very much for your attention and interest in our work, even though so far from you expertise.

My unforgetable time in this group was possible mainly due to singular people like Chen, which for a while composed with me the "Super Duo" - especialized in cell transfections and in situ hybridizations; Angelito, the most easy going person to work with (except during the moving); Daddy Paul, the best example of a dutch colleague (discrete, but direct); and Maaike, which showed a quiet, but always there, friendship. Maaike, I will be always in debt with you and Yuri with the help with the layout and cover of this thesis, I did nothing!!! I like that! Good luck in USA and I hope we will be always in contact.

Paula and Hugo, you two are the most preccious friends I made here. Thank you, dank u wel, and muito obrigada for everything you did to help me and to support me. Of course, our happy moments of dinners, trips and movies will be kept in my memories.

Beside my colleagues, some people were very important in my ducth life for making me feel at home in Utrecht. Rosa and Angie, there are no words to exactly describe how unique your friendships are for me. Thanks for everything, all the moments that you know. My life here would never be the same if I had not met you two and Stan, Lucas and Marteen. Ellen, Vanessa and Esther, thanks for the good, happy and emotional moments that girls like to share. Eric, Melvin and Phil, I will never forget our memorable international dinners (only for VIPs).

I also want to express my gratitude to Fernando, who supported me in this PhD from the first day and came with me to this other side of the world and never complained about the difficulties.

Finally my special thanks to my family, Irajá, Judith, Octávio and Janina. I will be always in debt with them, considering all the support, care and unconditional love I received. I can not imagine how it would be possible to finish this PhD without the love and respect from this four.

Paieco, mãe, mano e pipoca, eu não tenho palavras para expressar minha gratidão por tudo por tudo o que vocês fizeram por mim. Todo o apoio e amor que eu recebo de vocês, além de me engrandecer como pessoa, nos une cada vez mais, e assim compomos uma famíla com respeito mútuo e amor incondicional. Obrigada por tudo MESMO!

CURRICULUM VITAE

I was born on the 14th of October 1976 in Corumbá (Mato Grosso do Sul), a city in the middle of the Brazilian Pantanal. Daughter of veterinarians and a passion for horses, I graduated in the Veterinary Faculty of the Federal University of Mato Grosso do Sul, Campo Grande, at the end of 1999. After working for one year on clinic and reproduction of horses in Corumbá and near farms, I went to Belo Horizonte to take a master degree in Animal Reproduction at the Veterinary School of the Federal University of Minas Gerais in the laboratory of Prof. Dr. Luiz Renato de França, where I studied wild boar spermatogenesis for two years.

Upon completion of the master degree I returned to Campo Grande where I worked for the University of Dourados during one and a half years as assistant professor of the following chairs: Histology and Embriology of Domestic Animals, Physiology of Reproduction of Domestic Animals, and Scientific Methodology for the courses of Veterinary Medicine and Biochemistry and Pharmacy. In 2004 I was indicated, by Prof. Dr. França, for a PhD position available in the Endocrinolgy Group of Prof. Dr. Dirk de Rooij at the Utrecht University under the supervision of Dr. Rüdiger W. Schulz, who I briefly had met before in a Brazilian conference. The project would be developed in collaboration with the Group of Reproduction and Growth, led by Dr. Geir Lasse Taranger, at the Institute of Marine Research in Bergen, Norway. After four years I am very glad to have developed this project on puberty of male cod, especially for learning so much about this marvelous and diverse world of fish spermatogenesis.

LIST OF PUBLICATIONS

Almeida FFL, Kristoffersen C, Andersson E, Mittelholzer C, Karlsen Ø, Taranger GL, Schulz RW. Pituitary gonadotropin and testicular gonadotropin receptor expression in Atlantic cod (*Gadus morhua*, L.) during the first reproductive season: effects of photoperiod modulation. In preparation.

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