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Photoperiod manipulation can stimulate or inhibit pubertal testis maturation in Atlantic salmon (*Salmo salar*)[1]

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Abstract

Early puberty affects in particular males in commercially farmed European fin-fish species, resulting in reduced growth and feed conversion, and the potential problem of unwanted genetic impacts on wild stocks. Controlling photoperiod can be an effective way to prevent early sexual maturation. Combining national and international resources provided the means to embark on a research program to improve our knowledge on the mechanisms of pubertal testis development in a number of finfish species, including the Atlantic salmon. In addition to its applied relevance, a study aiming to elucidate how the initiation of spermatogenesis is regulated in a vertebrate is of general interest. We report here that exposure of previously immature male salmon to constant light (LL) starting shortly after the winter solstice reduces the incidence of male puberty from 100% to 60%. In the 40% of animals where entrance into puberty was blocked, histological analysis of testis tissue indicated that an even lower level of activity results from LL exposure than is found in testes of animals before entering puberty. In the 60% of males maturing under LL treatment, testis development was significantly accelerated compared to the control group exposed to natural light (NL). Hence, photoperiod manipulation of puberty generates experimental groups with diverging states of testis development that are excellently suited to study the endocrine regulation of testis development (e.g. circulating hormone levels; hormone and hormone receptor gene expression in hypothalamic, pituitary, and testis samples).

Keywords: Atlantic salmon, precocious male puberty, photoperiod manipulation, testis growth, spermatogenesis.

Introduction

In many finfish species introduced in aquaculture, the process of sexual maturation adversely affects somatic growth since energy resources are shifted from growth-related to reproductive processes. The impact of this shift is reflected by the large increases in gonad weight during sexual maturation in many seasonally reproducing fish species, or by considering the energy demand of extended spawning migrations such as known from salmon or eel. As regards gonad weight and the gonado-somatic index (GSI; gonad weight expressed as percentage of the total body weight), increases are observed in several species from ~0.1% in prepubertal animals or in adults out of the reproductive season, to ~10% in males and ~25% in females, upon reaching full maturity. Next to the adverse effects on growth, sexual maturation in aquaculture production facilities can result in unwanted genetic impacts on natural stocks, via the release into the environment of fertilized eggs, or via escapees (Jonsson and Jonsson, 2006). Finally, in many salmonid species, such as in the Atlantic salmon (*Salmo salar*), the life cycle is characterized by a juvenile period in freshwater, followed by the main somatic growth period in the marine environment, and a subsequent, spawning-associated migration back into the freshwater environment. In addition to the energetic requirements of migration *per se*, the osmoregulatory system is adjusted – under the influence of sex steroid hormones – to moving between a hyper- (marine) and hypo-osmotic (freshwater) environments. Since salmon aquaculture sites are situated in seawater, the maturation-associated loss of osmoregulatory capacity in seawater brings about animal welfare problems, including high mortality if kept is in seawater throughout maturation (Taranger, 1993). Taken together, sexual maturation entails a range of problems that compromise sustainability aspects of aquaculture biotechnology.

Precocious puberty is a male-biased phenomenon

Having said this, it becomes particularly urgent to deal with a problem that is observed with many species when introduced into aquaculture with feed levels that allow rapid growth, namely the precocious start of puberty. In cultured cod and salmon, for example, puberty is observed 1-4 years earlier than in feral animals (for example Godø and Moksness, 1987; Svåsand *et al*., 1996). This phenomenon is particularly relevant and observed rather early during ontogenesis in males in these species, probably related to the lower energetic requirements of male compared to female gonad maturation. In context with the above-mentioned problems brought about by puberty, in salmon this can lead to the loss of a large proportion of males before the animals have reached a marketable size (Hansen *et al*., 2000).

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Previous work has shown that although restricted feeding can reduce the incidence of precocious puberty, the negative effect on growth limits the feasibility of this approach in both salmon and cod (*e.g*. Thorpe *et al*. 1990; Thorpe, 1994; Karlsen *et al*., 1995). A more effective means showing fewer side effects seems to be the exposure of animals to additional or continuous light starting around the winter solstice in the case of Atlantic salmon (Taranger *et al*. 1991; 1998; Hansen *et al*. 1992; Oppedal *et al*. 1997, Porter *et al*. 1999). However, the response of the animals to light treatment shows unpredictable elements: The percentage of males inhibited to enter puberty varies between years and sites, and different salmon strains can show different response tendencies (Taranger *et al*., 1999; Endal *et al*., 2000).

Endocrine aspects of male puberty and the initiation of spermatogenesis

Similar to the situation in other vertebrates, also in fish the brain-pituitary system is the master control unit for reproductive processes, including puberty (Schulz and Goos, 1999; Okuzawa, 2002). It appears reasonable to assume that light-induced modulation of the pubertal activation of this system, via the pineal organ and/or the lateral eyes, eventually is conveyed to neuroendocrine neurons producing gonadotropin-releasing hormone (GnRH). Ensuing changes in pituitary follicle-stimulating hormone (FSH) and luteinizing hormone (LH) release will change Sertoli and Leydig cell activities, respectively, resulting in changes in testicular physiology. This was shown in recent reports on the GnRH, gonadotropin and androgen levels in male sea bass (*Dicentrarchus labrax* L.) during normal and photoperiod-manipulated entry into puberty (Rodriguez *et al*., 2004; 2005). However, little is known in fish – as in other vertebrates – about exactly what processes are targeted by, for example, FSH and androgens in the testis, or how the gene networks are organized that respond to these endocrine signals, either in Sertoli cells, or – possibly after triggering Sertoli cell paracrine signaling – in germ cells. Next to these basic research questions requiring attention, detailed information is also missing as regards the effects of light treatment on salmon spermatogenesis. Most studies so far have recorded testicular weight, or the presence of readily releasable sperm as parameter reflecting testis development (*e.g*. Endal *et al*. 2000), but no information is available on the timing of spermatogonial proliferation or entry into meiosis, for example, or the effects of light treatment thereon. Clearly, morphological, physiological, and molecular data on the timing and regulation of the developmental processes constituting (precocious) puberty on the testicular level are required to generate a basis, on which to design protocols that will allow a better predictability of treatments aiming at controlling puberty.

The fact that testis weight stays low in light-inhibited animals suggests that the undifferentiated spermatogonia populating the prepubertal testis, do not proliferate and differentiate, in contrast to those in testis of control animals that do undergo puberty. Investigating this experimental model therefore is not only of potential practical use for aquaculture biotechnology, but is interesting from a basic research point of view, since it allows studying a model, in which an environmentally relevant cue (photoperiod in this case) can modulate – via the endocrine system and its messenger molecules – testicular activity such that a testis containing predominantly undifferentiated spermatogonia can be induced, or inhibited, to switch to spermatogonial proliferation/differentiation. Given the evolutionary conservation of many signaling cascades, this model may provide interesting results for vertebrates in general, and allows bridging different levels of biological integration, from ecology (photoperiod, availability of food), via organismal/cellular physiology (endocrine/paracrine signaling), to the role of messenger molecules in modulating cellular developmental pathways.

Materials and methods

In order to work on this approach, national and international resources have been bundled to support a research program with the initial aim to generate a set of analytical tools for a number of finfish species relevant in aquaculture. This has involved the cloning of GnRH receptor and pituitary gonadotropin subunits genes and development of respective real-time quantitative polymerase chain reaction (rtqPCR) assays; the set up/adaptation of immunoassays for circulating gonadotropic and sex steroid hormones; the cloning and pharmacological characterization of gonadotropin and sex steroid receptors, again including the development of respective rtqPCR assays to quantify gene expression. Following tool generation, they are/will be used analyzing samples from animals exposed to photoperiod regimes that modulate the timing of male puberty (see below). Development and characterization of these tools will be described in detail elsewhere. The remainder of this contribution deals with a trial, in which male Atlantic salmon were exposed to a photoperiod treatment known to modulate entrance into puberty.

Light treatment and sampling

Previously immature salmon were maintained in sea cages at the Institute of Marine Research (Matre, Norway) under ambient light conditions (NL; 61°N) 19 months until the start of the trial (January 2004), when the animals had been in seawater cages for 18 months and were 3 years old (January 2004). An initial control sample was collected on January 8, 2004. Starting February 1, half of the animals were exposed to additional constant light (LL), while the other half

remained under NL conditions. Samples were then collected at 4 time points: February 18, March 19, April 25, and June 11. In June, maturation was clearly visible in all males of the NL group and the experiment was terminated. In subsequent trials (not reported here), maturing animals were moved in June from sea cages (marine environment) to a freshwater environment to imitate entrance into rivers during the spawning migration (required in context with the maturation-induced change of osmoregulatory capacity). In freshwater, a further, major increase in testis weight occurs towards full maturation in spawning animals, reaching GSI levels ca. 5 times above those recorded in maturing males in June.

For tissue and blood sampling, the fish were netted from the cages, immediately anaesthetized with 6ppt metomidate (Syndel, Victoria, B.C., Canada), weighed (total body weight), blood (5 ml) was collected in heparinized syringes from the caudal veins and gonads excised and weighed. The gonado-somatic index was calculated as: GSI (%) = gonad weight (g) *100 / total body weight (g). Tissue samples were either shock-frozen in liquid nitrogen and then stored at –80ºC, or fixed for different purposes. For routine histological analysis, a testis tissue fragment was fixed in phosphate buffered 4% paraformaldehyde and 2% acetic acid, and dehydrated and embedded in paraffin wax, according to conventional techniques; 5 µm sections were stained with hematoxylin and eosin.

The progress through spermatogenesis was recorded by identifying the furthest progressed cohort of germ cells and assigning the animal to one of the following classes: (i) predominantly early spermatogonia, (ii) regular presence of late spermatogonia, (iii) presence of spermatocytes; (iv) presence of spermatids; (v) presence of sperm.

Results

In the start control sample (Jan 8) an average GSI value of 0.05 ± 0.01 was recorded in the males. At the 2nd sampling 6 weeks later (Feb 18), 2.5 weeks after the start of exposure (Feb 1) of half of the fish to LL, we recorded a split in both, the NL and the LL groups. About half of the males in both groups showed GSI values of at maximum 0.05 (see Fig. 1; 4 of 9 animals in the NL group designated NL_{low}; 4 of 10 animals in the LL group designated LL_{low}), similar to the level recorded in January, whereas the remaining animals showed significantly higher GSI values of at least 0.07 and were assigned to groups designated NL^{high} or LL^{high}, respectively; this separation was confirmed by histological analysis (see below). A similar situation was recorded in March, although the difference between the subgroups showing low or high GSI values, respectively, was more pronounced than in February (Fig. 1). Analysis of the April and June samples revealed that in the NL group, all males except one in April had been recruited into maturation. In LL-exposed fish, on the other hand, the light treatment blocked testis growth in ca. 40% of the males, while in the remainder of LL-treated males, testes weight increased to levels significantly higher than those found in the maturing animals of the NL group.

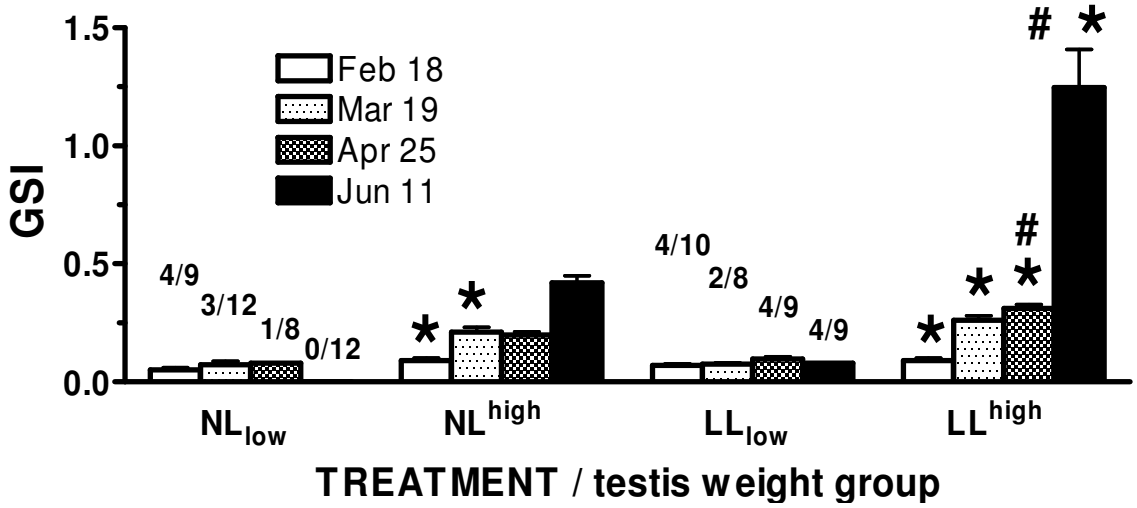

Figure 1. Fish were exposed to normal light (NL) or constant light (LL) starting February 1, and sampled at the indicated dates. The numbers indicate the incidence of maturation: the first digit gives the number of non-maturing males, the second digit the total number of males at this sampling date; *significant difference ($p<0.05$; ANOVA followed by a Fisher least significant difference test) to the respective NL_{low} or LL_{low} group, # - significant difference to the respective NL^{high} group.

In general, spermatogenesis in fish (and amphibians) takes place in cysts within the seminiferous tubules. The spermatogenic cysts form when Sertoli cells enclose a single primary spermatogonium (Pudney, 1993). The germ cells derived from a single primary spermatogonium then divide synchronously to constitute an isogenic germ cell clone that is bordered by the cytoplasmic extensions of a single layer of Sertoli cells. Hence, in cystic spermatogenesis, a Sertoli cell 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. To accommodate the increasing number of germ cells, the cyst-forming Sertoli cells proliferate, in particular during the spermatogonial phase (Schulz *et al*., 2005). Spermiation, the release of mature germ cells by Sertoli cells, is achieved by breaking cell-cell contact between Sertoli cells to open the cysts, such that cyst lumen and lumen of the seminiferous tubules unify.

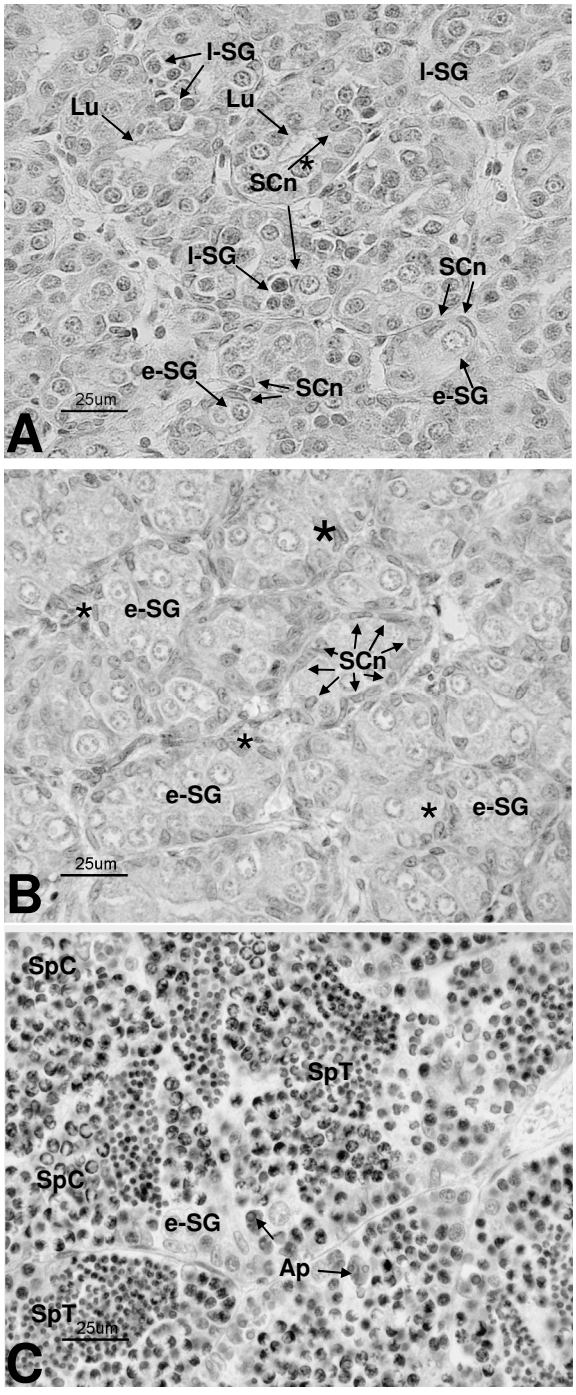

Figure 2. Hematoxylin-stained, 5-µm sections from Atlantic salmon testis. A – Salmon testis from NL^{high} group sampled February 18, showing signs of maturation, such as late spermatogonia (l-SG) next to early spermatogonia (e-SG); Sertoli cell nuclei (SCn) are found in the periphery of the tubules but also among the germ cells (SCn*). B – Salmon testis from LL_{low} group sampled March 19, showing early spermatogonia as predominant/only germ cell type, while Sertoli cell nuclei are mainly found in the periphery of the seminiferous tubules that do not show lumen formation; asterisk indicates a cluster of Sertoli cell nuclei. C – Salmon testis from LL^{high} group sampled June 11, showing in addition to spermatogonia many spermatocytes (SpC)in different stages of the first meiotic prophase but also numerous spermatids (SpT); apoptotic (Ap) cells were observed in all rapidly growing testes. Bar = 25 µm.

Histologically, the main difference between animals that just started to mature and those still immature was the ratio between early and late spermatogonia, the latter being more prominent after initiation of maturation in animals assigned to the NL^{high} group (Fig. 2A). Early spermatogonia are large cells occurring singly or in pairs or small groups, showing little heterochromatin, a single nucleolus, and a relatively large but pale cytoplasmic area. Late spermatogonia are smaller cells, occur in groups, and show more heterochromatin and a more darkly staining cytoplasm. The maturing testes also showed beginning of lumen formation in the seminiferous tubules. An interesting difference was recorded between males in the NL group that did not yet start to mature (similar to Fig. 2A but with less late spermatogonia) and males in the LL group where maturation was blocked (Fig. 2B). The latter animals, constituting the LL_{low} group, were characterized by the very low number/absence of late spermatogonia and by the localization of many Sertoli cells nuclei in the periphery of the seminiferous tubules close to the basement membrane, such that the Sertoli cells' nucleoplasm formed a dark ring that surrounded pale, undifferentiated, large spermatogonia. In these testes, we also found groups of apparently inactive Sertoli cells with their nuclei clustered rather close to each other, indicating that the cytoplasmic volume was small (Fig. 2B). The main difference between maturing animals of the NL^{high} and LL^{high} groups, respectively, was the earlier (already in the April 25 sampling) or more frequent (in the June 11 sampling) appearance of postmeiotic cells (Fig. 2C) in the LL^{high} group.

Discussion

The present trial confirmed that exposure to LL is a powerful means to modulate pubertal development in first time maturing male Atlantic salmon (Oppedal *et al*. 1997; Taranger *et al*. 1998, Porter *et al*. 1999). All males kept under NL conditions were recruited into maturation, while testis growth and spermatogenesis was suppressed in 40% of the LL-exposed males. We show here for the first time that LL-induced suppression of maturation is not only arresting testis development at an early stage of maturity but seems to reduce the level of activity further below the initial control stage recorded in the January sampling. Histologically, this is reflected in the absence of germ cells other than early spermatogonia and undifferentiated, apparently inactive Sertoli cells. The changes in spermatogonial morphology during their development towards meiosis, such as decreasing nuclear size or increasing amount of heterochromatin and cytoplasmic stainability, have been described in other fish species as well (Billard, 1969; Matta *et al*., 2002). Exposure to LL is able to keep animals for a prolonged period in this basal state of activity, since the combination of traits has been recorded from the February until the June sampling. It would be interesting to investigate if relieving LL-inhibited males from the light exposure would allow these males entering puberty, and if discontinuation of LL exposure at different times between winter and summer solstice would result in different effects.

Worthy of note was the coincidence of the proportion of maturation in the NL group in Feb (4 immature and 5 maturing males) and the maturational response to LL treatment where, overall, in 40% of males testis development was arrested, while 60% displayed an accelerated testis growth, entry into meiosis, and presence of postmeiotic cells. It would appear that at the period when day length is at the annual minimum, males can be in one of two physiological conditions: a condition that prevents, and a condition that allows, responding to LL exposure by rapid pubertal maturation.

Our future work is directed to two main aims. In the first instance to improve our knowledge on the regulatory background of modulating testis maturation with regard to the identity and function of hormone or growth factor target genes responsible for shutting down or accelerating testis development. Secondly, in a broader sense, we wish to characterize the elements that constitute the physiological conditions enabling either a maturational, or an inhibitory response of the individual. These aims will be approached initially by examining candidate gene expression in brain, pituitary, and testis tissue samples, by quantifying circulating hormone levels, and by further morphological analysis of the developmental processes in the testis. In the long run, specific, well-characterized individuals will be examined by large-scale gene expression analysis, for example applying the GRASP 16k cDNA microarray (http://web.uvic.ca/cbr/grasp/; von Schalburg *et al*., 2005) that is used at present in our laboratory for studies on the skeletal system.

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