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Continuous light affects onset of puberty and associated changes in pituitary gonadotropin subunit transcript levels, and plasma estradiol-17β and testosterone levels in Atlantic cod (*Gadus morhua* L.) females

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ABSTRACT

To gain more insight in photoperiod modulation of timing of puberty and its endocrine control in female Atlantic cod, ovarian development, plasma sex steroid profiles and mRNA transcript levels of the pituitary gonadotropin subunits *fshb*, *lhb* and *gpa* were monitored under four different photoperiod regimes over 16 months. Pre-pubertal cod were subjected to ambient (NL group) or continuous (LL group) light in light-proof seawater tanks from June in their second year of life. On December 21, half of the NL group were transferred to continuous light (NL–LL group), and half of the LL group was moved to ambient light (LL–NL group). The fish were maintained under these conditions until November the following year. All females in the NL group entered puberty at two years of age as indicated by increasing gonadosomatic index (GSI), oocyte diameter, pituitary *fshb*, *lhb* and *gpa* mRNA levels, as well as plasma testosterone (T) and estradiol-17β (E2) levels. The NL females had hyaline oocytes or spent ovaries indicative of spawning in January to April, and subsequently started a new wave of maturation by October. Low GSI, oocyte diameter, pituitary *fshb*, *lhb* and *gpa* mRNA levels, and low plasma E2 and T levels demonstrated arrested maturation in most females in the LL group during the normal spawning season, while some maturing fish were noted towards the end of the experiment. Compared to LL females, NL females showed elevated levels of T by July in the first year of the study, *fshb* by September, E2 by October, and *lhb* by December. This suggests that the LL arresting effect initially was mediated by reduced Fsh signaling and lowered plasma T levels during the period of previtellogenic growth in summer and early autumn, followed by low E2 levels and inhibition of onset of vitellogenesis in late autumn and early winter. LL–NL treatment delayed puberty onset and completion by 5 months, while NL–LL resulted in earlier completion of the spawning season compared with NL. The accelerating and delaying effects observed on puberty and spawning in the LL–NL and NL–LL groups, respectively, were paralleled by similar shifts of the seasonal profiles of *fshb*, *lhb*, *gpa*, E2 and T.

1. Introduction

Farmed Atlantic cod in southern Norway usually reach puberty at the age of two years (Godø and Moksness, 1987; Karlsen et al., 1995). Sexual maturation is associated with a rapid increase in gonad weight, typically peaking in January–February, at around 10 and 15% of total body weight in recruit spawning male and female farmed Atlantic cod, respectively (Davie et al., 2007; Karlsen et al., 1995; Taranger et al., 2006). This early puberty is a major problem in cod farming due to negative effects of maturation on appetite, somatic growth, food conversion efficiency, production time, survival and fish welfare (Taranger et al., 2010).

Photoperiod manipulation techniques have been applied to delay or inhibit this early maturation in farmed cod (Almeida et al., 2009; Cowan et al., 2011; Davie et al., 2007; Hansen et al., 2001; Karlsen et al., 2006a, b). However, these attempts have only been partially successful when applied in commercial scale sea cages (Skulstad et al., 2013; Taranger et al., 2006). Additional knowledge about how photoperiod modulates puberty entry and completion and the endocrine mechanisms controlling these events are therefore needed for refinement of such techniques for use in sea cage farming.

Ovarian development is under endocrine control through the brain–pituitary–gonad (BPG) axis in Atlantic cod as in other vertebrates. Onset of puberty in female Atlantic cod is correlated with increasing transcript

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levels of the pituitary gonadotropin subunit follicle-stimulating hormone-beta (*fshb*), while the pituitary transcript levels of the gonadotropin subunit luteinizing hormone-beta (*lhb*) increase during the final stages of oocyte maturation and ovulation (Mittelholzer et al., 2009). It is assumed that follicle-stimulating hormone (Fsh) stimulates production of the sex steroids testosterone (T) and estradiol-17β (E2) in teleost fishes, and that E2 in turn induces production of vitellogenin in the liver to fuel vitellogenesis (reviewed by Nagahama and Yamashita, 2008).

Continuous light (LL) was effective in arresting testicular development in most Atlantic cod males when applied in light-proof tanks with no access to ambient light (Almeida et al., 2009). This arresting effect of LL was paralleled with reduced levels of pituitary *fshb* transcripts suggesting that reduced Fsh signaling played a major role in mediating the observed arresting effects of LL in males (Almeida et al., 2011). When LL was applied at different time-points in the year, this modulated the seasonal patterns of pituitary *fshb* and *lhb* transcript levels as well as plasma testosterone and 11-ketotestosterone levels. The current study presents data from the females of the same experiment, covering puberty and initiation of maturation in repeat spawning females, in order to analyze effects of LL applied at different time-points in the seasonal cycle on puberty onset and completion, and initiation of maturity in repeat spawning female Atlantic cod maintained in seawater tanks. This was done to increase our understanding on how LL affects the molecular and endocrine regulation of maturation in Atlantic cod females.

2. Materials and methods

2.1. Animals, photoperiod regimes and samples

Pre-pubertal Atlantic cod (Norwegian coastal cod origin) 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°N). The cod originated from larvae hatched in March 2003 and 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 libitum. The tanks were supplied with seawater pumped from 168 m depth and water temperature ranged from 7.4 to 9.4 °C (mean ± SD, 8.1 ± 0.3 °C) during the experimental period. All fish were treated and euthanized according to the Norwegian National Legislation for Laboratory Animals (1996).

From July to December 2004 the animals were divided into two experimental groups, each in 6 replicate tanks. One group was reared under natural light (NL) (tanks covered with 70% light reducing shading nets), while the other group was exposed to continuous light (LL) in light-proof tanks. The light in each LL tank was supplied by a 70 W metal halide lamps (Phillips MHW-TD 70 W light bulbs), giving an integrated irradiance of 5.5 e^{-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 (NL group); 2. LL throughout (LL group); 3. six months of NL followed by eleven months of LL (NL–LL group); and 4. six months of LL followed by eleven months of NL (LL–NL group).

Every month, 30 animals were sampled randomly from each group, among which 11–20 were females. Blood was withdrawn from the caudal vein with a cold, heparinized syringe and plasma was separated by centrifugation for 3 min at 3000 rpm. After centrifugation, plasma was immediately frozen on dry ice and stored at −80 °C until analysis. Total length (±0.5 cm) and body weight (±1 g) were recorded, and the fish killed by cutting the medulla oblongata. The entire pituitary was immediately excised, wrapped in aluminum foil and snap-frozen by immersion in liquid nitrogen, and stored at −80 °C for later RNA extraction and cDNA synthesis. Liver and gonads were excised and weighed (±0.5 g), and an ovarian sample from the middle of the right lobe was fixed in buffered formalin for oocyte diameter determination as described below, and gutted weight recorded (±1 g). Gonadosomatic index (GSI) was calculated as gonad weight·100·body $weight^{-1}$.

There was no statistically significant difference in GSI between animals from the six or three replicate tanks from the same light treatment, allowing data to be pooled according to the photoperiod regime. The mortality was low with an overall loss of 9% over the 17-month period. The sex ratio was 50.7% females (integrated for all the samplings).

2.2. RNA extraction and reverse transcription

Pituitaries stored at −80 °C were immediately transferred to cold Tri Reagent (Sigma-Aldrich, Oslo, Norway). After homogenization in a FastPrep microfuge tube containing Lysing Matrix D ceramic beads, total RNA was extracted by the acid phenol–guanidinium thiocyanate method followed by purifying the RNA through a column (RiboPure kit, Ambion/Applied Biosystems, Oslo, Norway), and quantified by spectrophotometry. Random-primed cDNA was synthesized from exactly 500 ng RNA using a Reverse Transcription Core Kit (RT-RTCK-05, Eurogentec, Seraing, Belgium), and diluted 1:10 in nuclease-free H_2O before use in the quantitative real-time PCR. For a choice of samples, RNA quantity and quality were also determined by capillary electrophoresis using the Lab on a Chip technique (Agilent 2100 BioAnalyzer, Santa Clara, CA, USA) in accordance with the manufacturer's instructions on the RNA 6000 Nano Labchip.

2.3. Quantitative real-time PCR (qPCR)

The qPCR assays for Atlantic cod gonadotropin beta subunits (*fshb* and *lhb*), the common alpha subunit (*gpa*) and for the reference gene elongation factor 1 alpha (*ef1a*) have been described in detail previously (Mittelholzer et al., 2007, 2009). Primers and TaqMan hydrolysis probes (listed in Table 1) were designed with Primer express software (Applied Biosystems, Oslo, Norway), according to the manufacturer's guidelines. Probes span exon–exon boundaries in the mRNA sequence to prevent genomic DNA amplification. Optimal primers and probe levels were then determined following Applied Biosystems guidelines, and serial dilutions of cDNA were tested to assess the quality of the qPCR. The qPCR assays were performed in duplicate, using 96-well optical plates on an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Oslo, Norway) using default settings. For each 25 μl PCR reaction 2.5 μl cDNA was mixed with 200 nM hydrolysis probe, 900 nM sense primer, 900 nM antisense primer in 1× TaqMan Universal PCR Master Mix (Applied Biosystems, Oslo, Norway; see Table 1 for the primer/

Table 1
Primers and probes of the quantitative real-time PCR.

Name	Sequence (5′ → 3′)	Position
Gpa:		
acgpa_q1	CTG TGT CGC ATC ACG TAG CA	258–277
acgpa (probe)	6FAM-TCC GAC CAA GGT TAC C-MGB	279–294
acgpa_q2	CCA ACT CTG TGT TTC CGT TTC C	319–298
Fshb:		
acfshb_q1	TCA GAA CCG AGT CCA TCA ACA C	107–128
acfshb (probe)	6FAM-CCA TGT GTG AGG GCC-MGB	131–145
acfshb_q2	GGT CCA TCG GGT CCT CCT	172–155
Lhb:		
aclhb_q1	CCT CTC CGT GGC GCT GG	36–52
aclhb (probe)	6FAM-CGC CCC TGG GAG CAG CCT ATC AGC	62–85
aclhb_q2	GGG ACA GCC CTT CTT CTC CA	141–122
Ef1a:		
aceef1a_q1	GCC CCT CCA GGA CGT CTA C	
aceef1a (probe)	6FAM-AGA TCG GCG GTA TTG-MGB	
aceef1a_q2	ACG GCC CAC GGG TAC TGT	