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Quantification of gonadotropin subunits GPα, FSHβ, and LHβ mRNA expression from Atlantic cod (*Gadus morhua*) throughout a reproductive cycle

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ABSTRACT

To elucidate the role of the gonadotropins in Atlantic cod (*Gadus morhua*), complete coding sequences with partially or fully un-translated regions for the three subunits GPα, FSHβ, and LHβ were determined. The sequences of the corresponding genomic loci were also determined, allowing the design of mRNA-targeting quantitative PCR assays. Relative expression was analyzed during a complete seasonal sexual maturation cycle in Atlantic cod females. Increasing levels of *lhβ* mRNA were observed during gonadal growth, peaking at spawning in February–March which corresponds to maximum gonadosomatic index. In contrast, both *gpα* and *fshβ* gradually increased to a peak in December, two months before spawning started, and decreased in January just prior to spawning. Both mRNAs increased again and remained high during the spawning season, with a decline at the end of the spawning period, a further decrease in spent females, followed by a new gradual increase concurrent with the start of the next reproductive cycle. In addition to its role in vitellogenesis prior to spawning, FSH seems to have additional functions during the spawning period, possibly related to vitellogenesis that runs in parallel with final oocyte maturation and ovulation of the multiple batch spawner Atlantic cod.

1. Introduction

A stable and predictable supply of gametes of defined quality on one side, and the prevention of precocious maturation on the other side, are crucial steps in the successful farming of Atlantic cod (*Gadus morhua*). The two gonadotropin hormones, follicle-stimulating hormone (FSH), and luteinizing hormone (LH), are major players in the brain–pituitary–gonadal axis controlling puberty and reproduction. These pituitary hormones are heterodimeric, non-covalently bound glycoproteins composed of a common α-subunit and a hormone-specific β-subunit (Pierce and Parsons, 1981). Both the α- and β-subunits of the gonadotropin hormone gene family diverged from a common ancestral gene about 927 million years ago (Li and Ford, 1998), and the existence of all three subunits have been reported for all investigated species of jawed vertebrates (Gnathostomes), including a considerable number of fish species, as summarized by Sower et al. (2006). Together with thyroid-stimulating hormone (TSH), they form the vertebrate glycoprotein hormone family (Pierce and Parsons, 1981).

It is generally accepted that gonadotropic regulation of gametogenesis in female fish spawning single batches of eggs is characterized by increasing plasma FSH levels during oocyte growth and peak levels of LH in plasma at final maturation and ovulation (Naito et al., 1991; Prat et al., 1996; Hassin et al., 1999; Gomez et al., 1999; Kumar & Trant, 2004; Kim et al., 2005). In contrast, a simultaneous increase in both *fshβ* and *lhβ* mRNA levels in the pituitary has been reported in several multiple batch spawners during the advancement of the gonadal cycle (Elizur et al., 1996; Yoshiura et al., 1997; Jackson et al., 1999; Sohn et al., 1999; Kajimura et al., 2001; Mateos et al., 2003; Weltzien et al., 2003; Meiri et al., 2004). Consequently, both gonadotropins are likely required to stimulate the development of heterogeneous batches of follicles in multiple spawning fish. However, gonadotropin subunit transcript levels and plasma levels are not always correlated, and data from expression studies should be interpreted with caution. In addition, FSH and LH might not only bind exclusively to their corresponding receptor, as it is the case in mammals, but also to the other gonadotropin receptor. There are an increasing number of reports on promiscuous ligand-receptor binding in fish (Yan et al., 1992; Miwa et al., 1994; Oba et al., 1999a,b; Bogerd et al., 2001; Vischer et al., 2003; So et al., 2005). Differential stimulation by FSH and LH might also be achieved by spatio–temporal regulation of their receptor expression (Oba et al., 2000; Kumar et al., 2001a,b; Hirai et al., 2002; Kwok et al., 2005). Recently the gonadotropin receptors FSH-R and LH-R have been characterized in Atlantic cod and their expression profiles have been studied with quantitative real-time PCR during a seasonal reproductive cycle showing that levels for FSH-R increased moderately during gonadal growth whereas those of LH-R

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showed a high peak at spawning, suggesting that FSH-R plays a major role in the growing phase and LH-R has its important role in final oocyte maturation and ovulation (Mittelholzer et al., 2009). However, the pharmacological characterization of the two receptors is still missing in cod, as well as precise information on their cellular localization in the theca and granulosa cells during the reproductive cycle. Moreover, it is not yet possible to quantify the circulating FSH and LH levels in Atlantic cod with a homologous detection assays, although the Atlantic cod FSH protein has recently been purified (B. Norberg and P. Swanson, unpublished results).

As a first step to elucidate the role of the gonadotropins during ovarian development and growth, final oocyte maturation and spawning in the multiple batch spawner Atlantic cod, the complete coding sequences with partially or fully un-translated regions for the three subunits GPα, FSHβ, and LHβ were determined. We developed quantitative real-time PCR assays for the analysis of expression of Atlantic cod *lhβ*, *fshβ* and *gpα* mRNA in individual pituitaries during a complete reproductive cycle.

2. Materials and methods

2.1. Animals and samples

Details about the animals used for sequence determination and quantitative real-time PCR, and the sampling protocols have been described previously (Mittelholzer et al., 2009). Briefly, larvae of Norwegian coastal cod (*Gadus morhua*) were hatched in the laboratory and first fed on natural zooplankton in a semi-enclosed seawater pond according to the method of Øiestad et al. (1985) before transfer to the experimental tanks or net pens, where they were fed a commercial dry pellet diet *ad lib*. For sequence determination, fish kept at ambient photoperiod (60°N) and water temperature were killed in accordance with Norwegian regulations for fish sacrifice, pituitaries were collected immediately and snap-frozen in liquid nitrogen before storage at −80 °C. For quantitative real-time PCR, sixteen months old male and female Atlantic cod were kept under ambient light (60°N) in tanks supplied with seawater pumped from 168 m depth (temperature ranged from 7.4 to 9.4 °C (mean ± SD = 8.1 ± 0.3 °C) during the experimental period) during their first reproductive cycle (i.e. puberty) at 2 years of age and into the start of the second reproductive cycle. At monthly intervals from September 2004 until October 2005, 13–20 females were randomly collected from the tanks and stunned by a blow to the head. Body weigh was recorded, and gonads excised and weighed. Gonadosomatic index (GSI) was calculated as; GSI (%) = gonad weight*100/body weight.

2.2. RNA extraction and cDNA synthesis

Samples stored at −80 °C were immediately transferred to cold Tri Reagent (Sigma-Aldrich, Oslo, Norway) to preserve RNA quality. After homogenization in a FastPrep microfuge tube containing Lysing Matrix D ceramic beads total RNA was extracted from 100 mg pituitary tissue by the acid phenol-guanidinium thiocyanate method followed by isopropanol precipitation. To facilitate cloning of mRNA, poly A^+ RNA was isolated using Dynabeads (Dynal, Oslo, Norway) from the total RNA described above, and cDNA synthesized following the manufacturer's instructions for the SMART kit (ClonTech, Saint-Germain-en-Laye, France). For quantitative real-time PCR individual pituitary samples were homogenized in a FastPrep microfuge tube containing Lysing Matrix D ceramic beads before total RNA was extracted by the acid phenol-guanidinium thiocyanate method followed by purifying the RNA through a column (RiboPure kit, Ambion), and quantified by spectrophotometry. Random-primed cDNA was synthesized from exactly 500 ng DNase-treated RNA using a Reverse Transcription Core Kit (Eurogentec, RT-RTCK-05), and diluted 1:10 in nuclease-free H_2O before use in the quantitative real-time PCR.

2.3. Assessment of RNA quality and extraction efficiency

For a number of randomly selected samples RNA quantity and quality were also determined by capillary electrophoresis using the Lab on a Chip technique (Agilent 2100 BioAnalyzer) in accordance to the manufacturer's instructions on the RNA 6000 Nano Labchip®. The chip also generates a quantitative measure of the amount of RNA loaded. RNA extraction efficiency was calculated by dividing the amount of extracted total RNA by the weight of the tissue used for extraction.

2.4. Cloning and sequence of cDNAs for Atlantic cod GPα, FSHβ, and LHβ

Degenerate primers were designed based on amino acid sequencing of purified Atlantic cod GPα protein or purified Atlantic cod FSHβ protein (B. Norberg and P. Swanson, unpublished results), and alignments of fish gonadotropin subunit nucleotide sequences, or alignments of fish gonadotropin subunit nucleotide sequences alone. These primers were applied to SMART 5′RACE-ready cDNA using the Advantage PCR system (ClonTech, Saint-Germain-en-Laye, France). Resulting PCR products were cloned into a pCR4-TOPO vector (Invitrogen, Paisley, UK) and cycle sequenced using an automated ABI PRISM Model 377 machine, based on incorporation of fluorescently labeled dideoxynucleotide terminators.

Based on the so-obtained sequence information for the three different fragments, 5′ and 3′-RACE (rapid amplification of cDNA ends) primers were designed. The RACE primers were then applied to SMART 5′RACE- or 3′RACE-ready cDNA synthesized from polyA+ RNA. The resulting fragments were cloned into a pCR4-TOPO vector (Invitrogen, Paisley, UK), and cycle-sequenced as described above. Consensus sequences from at least two clones (in the case of ambiguities at least three clones were sequenced) were assembled using the MegAlign software of the Lasergene package (DNASTAR). Primer sequences are shown in Table 1, and detailed amplification procedures are available from the authors on request.

Deduced amino acid sequences of *gpα*, *fshβ* and *lhβ* mRNAs were analyzed in silico using SignalP 3.0 (Emanuelsson et al., 2007) and NetNGlyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/) to predict signal peptides and putative N-linked glycosylation sites, respectively. Alignments of the mature polypeptide amino acid sequences were performed using the ClustalW multiple sequence alignment program (Thompson et al., 1994).

2.5. Determination of genomic sequences

Primers encompassing at least the whole coding sequences were applied to genomic DNA (a kind gift from Dr. Geir Dahle, Institute of Marine Research, Bergen, Norway). The resulting fragments were cloned into a pCR4-TOPO vector (Invitrogen, Paisley, UK) and cycle-sequenced as described above. Consensus sequences from at least two clones (in the case of ambiguities at least three clones were sequenced) were assembled using the MegAlign software of the Lasergene package (DNASTAR, Madison, WI, USA). Primer sequences are shown in Table 1, and detailed amplification procedures are available from the authors on request.

2.6. Quantitative real-time PCR

Primers and TaqMan fluorogenic probes specific for Atlantic cod GPα, FSHβ, and LHβ, respectively, were designed with Primer express software (Applied Biosystems, Oslo, Norway), according to the manufacturer's guidelines. All three assays span exon–exon boundaries in the mRNA sequence to prevent genomic DNA amplification. The primers were subjected to an initial testing by running them in a conventional PCR on both cDNA and genomic DNA. Only primer pairs resulting in amplification products of different sizes due to the