20 β -hydroxysteroid dehydrogenase and CYP19A1 are differentially expressed during maturation in Atlantic cod (*Gadus morhua*)

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Abstract

In order to better quantify the molecular mechanisms regulating final oocyte maturation and spawning, complete coding sequences with partially or fully untranslated regions for the steroidogenic enzymes, cytochrome P450 aromatase and 20 β -hydroxysteroid dehydrogenase, were cloned from ovaries of Atlantic cod (*Gadus morhua*). The nucleotide and amino acid sequences showed high homologies with the corresponding sequences of other fish species, and conserved features important for functionality were identified in both predicted proteins. The sequences of the corresponding genomic loci were also determined, allowing the design of mRNA-specific quantitative PCR assays. As a reference gene for the real-time RT-PCR assays, eukaryotic elongation factor 1 α was chosen, and the mRNA as well as the genomic sequence was determined. In addition, a real-time quantitative PCR assay for the 18S rRNA was adapted to be used in cod. Analysis of immature and maturing female cod from July to January respectively showed that the enzyme genes showed the expected quantitative changes associated with physiological regulation. However, mRNA for eukaryotic elongation factor 1 α , and to a lesser extent even 18S rRNA, showed variable expression in these samples as well. To find accurate standards for real-time PCR in such a dynamic organ as the cod ovary is not an easy task, and several possible solutions are discussed.

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Introduction

In teleosts, like in other vertebrates, puberty and reproduction are controlled by the brain–pituitary–gonadal axis. The gonadotropic hormones, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), secreted from the pituitary under control of gonadotropin-releasing hormones (GnRHs) released by the brain, play a central role. They act on somatic cells in the gonads, and thereby regulate gonadal biosynthesis of steroid hormones. During final oocyte maturation, a shift in steroidogenesis occurs in ovarian follicles, mediated by changes in gene expression of steroidogenic enzymes like ovarian P450 aromatase (encoded by the *cyp19a1* gene) and 20 β -hydroxysteroid dehydrogenase (20 β -HSD; Senthilkumaran *et al.* 2004). This is concomitant with a switch from predominantly FSH toward higher levels of LH, and leads to the production of primarily maturation-inducing steroid (MIS), instead of conversion of testosterone into estradiol (E₂), in the ovarian follicles. Whereas MIS is produced by the action of 20 β -HSD, the conversion

of androgens (C19) to estrogens (C18) is catalyzed by an enzyme complex containing P450 aromatase, product of the *cyp19* gene, and a flavoprotein NADPH-cytochrome P450 reductase (Simpson *et al.* 1994). The enzymatic activity of P450arom is thought to be the rate-limiting step in estrogen biosynthesis (Simpson *et al.* 1994) and changes in P450arom enzyme activity or expression of *cyp19* genes have been shown to be major regulators of the gonadal production of E₂ during reproduction and development (Chang *et al.* 1997). Numerous reports have therefore focused on the identification and characterization of *cyp19a1* genes in fish, but studies in periodic spawners, like Atlantic cod, are scarce (Van Nes *et al.* 2005), and to our knowledge none is dealing with events during final oocyte maturation and spawning.

Sequences for the carbonyl-reductase like 20 β -hsd gene are available only from a few teleost species (ayu, Tanaka *et al.* 2002; Japanese eel, Kazeto *et al.* unpublished; rainbow trout, Guan *et al.* 1999; Nile tilapia, Senthilkumaran *et al.* 2002; and zebrafish, Wang & Ge 2002), despite high similarity scores that should facilitate

cloning of homologous sequences from other species. The protein encoded by this gene belongs to the short-chain steroid dehydrogenase/reductase superfamily (Jörnvall *et al.* 1995) and catalyzes the NADPH reduction of carbonyl compounds and steroids. The gene is broadly expressed in a variety of fish tissues (Guan *et al.* 1999, Senthilkumaran *et al.* 2002, Wang & Ge 2002), and it is evident that the 20 β -HSD represents the key enzyme for the production of MIS (Tanaka *et al.* 2002).

In Atlantic cod (*Gadus morhua* L.) in Norwegian waters, vitellogenesis normally starts in October, while the peak in spawning activity occurs in February–March (Kjesbu 1994). Cod eggs are small, and vitellogenesis is a rapid process, which takes place in the months immediately prior to spawning, as well as during the spawning period (Kjesbu *et al.* 1991, 1996). The Atlantic cod is a periodic spawner, and each female can release 15–20 batches of pelagic eggs at 50–100-h intervals, during a period of 3–4 weeks (Kjesbu 1989). The endocrine regulation of the recruitment of oocyte clusters into final maturation is poorly understood in periodic spawners, and the MIS has not been conclusively identified in cod.

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. Optimal use of production facilities requires year-round access to high-quality juveniles, which can be achieved only by the manipulation of the time of spawning. On the other hand, unwanted early sexual maturation or puberty during on-growth will lead to economic losses due to decreased meat quantity and quality as well as increased susceptibility of the fish to diseases. To better quantify the events during puberty, final oocyte maturation, and spawning in Atlantic cod, we determined both mRNA and genomic sequences of two key genes, *cyp19a1* and *20 β -hsd*, and established mRNA-specific quantitative real-time PCR assays.

Materials and methods

Animals and sample collection

Larvae of coastal cod, hatched in spring 2000, were raised in a seawater pond (Parisvatnet) situated outside Bergen, Norway (Øiestad *et al.* 1985), and transferred to net pens at the same location in late autumn. The fish were transported to the Institute of Marine Research, Austevoll Research station (60°N) in July 2001 and maintained in a 12×12×5 m sea cage, at natural photoperiod and temperature. The temperature fluctuated through the year, between 5 °C (February–March) and 20 °C (August). At sampling, the fish were stunned by a blow to the head in accordance with Norwegian regulations for killing fish, and individual tissue samples were collected. The samples were either snap-frozen in liquid nitrogen

before storage at –80 °C, or stored in RNAlater (Ambion/Applied Biosystems, Oslo, Norway) at 4 °C for up to 1 month.

Cloning and sequence of Atlantic cod *cyp19a1* and *20 β -hsd* cDNA

Samples stored at –80 °C were cut into small pieces on dry ice before immediate transfer to cold Tri Reagent (Sigma–Aldrich) or Isogen (Nippongene, Tokyo, Japan), in order to preserve RNA quality. Samples stored in RNAlater were blotted with a wipe to remove excess liquid. Total RNA was extracted by the acid phenol–guanidinium thiocyanate method from 50–100 mg ovarian tissue, and polyA⁺-RNA was isolated using Dynabeads (Dynal, Oslo, Norway) according to the manufacturer's instructions. Random primed cDNA was synthesized with Superscript II (Invitrogen), and PCR was performed using degenerate primers designed for the amplification of a large part of the coding region of *cyp19a1* (Gen *et al.* 2001). The two resulting PCR products were cloned into a pGEM-T easy (Promega) and a pCR II-TOPO vector (Invitrogen) respectively, and cycle sequenced using an automated ABI PRISM Model 377 machine, based on the incorporation of fluorescently labeled dideoxynucleotide terminators.

Based on the sequences of the fragments, 5'- and 3'-rapid amplification of cDNA ends (RACE) primers were designed and applied to cDNA synthesized with the Marathon kit (ClonTech), according to the manufacturer's instructions. The resulting fragments were cloned into a pCR II-TOPO or pCR4-TOPO vector (Invitrogen) and cycle sequenced as described previously. Consensus sequences from at least two clones (in 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 and detailed amplification procedures are available from the authors on request.

Cloning and sequence of Atlantic cod *eef1 α* cDNA

Based on an alignment of 11 fish *eef1 α* sequences (AF321836, salmon; AF498320, trout; AF485331, carp; AY422992, zebrafish; AB056104, goldfish; AB013606, medaka; AB032900, yellowtail; AB075952, tilapia; AF467776, turbot; AF184170, gilthead seabream; AY190693, red seabream), primers were designed to encompass a region targeted by a published mRNA-specific salmon louse assay (Frost & Nilsen 2003). Amplification of both mRNA, from an ovarian cDNA library (Goetz *et al.* 2006), and genomic DNA (a kind gift from Dr Geir Dahle, Institute of Marine Research, Bergen, Norway) resulted in two fragments of different sizes, and both were cloned into a pCR4-TOPO vector before cycle sequencing as described previously. This allowed the

design of an mRNA-specific set of qPCR primers and probes, as well as 5'- and 3'-RACE primers. The latter were applied to cDNA synthesized with the SMART kit (ClonTech) according to the manufacturer's instructions. The resulting fragments were cloned into a pCR II-TOPO or pCR4-TOPO vector (Invitrogen) and cycle sequenced as described previously. 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 and detailed amplification procedures are available from the authors on request.

Determination of genomic sequences

Primers encompassing the whole coding sequences were applied to genomic DNA, either kindly provided by Dr Geir Dahle, Institute of Marine Research, Bergen, Norway or extracted from ovary and liver respectively by a modified proteinase K (PK) method. Briefly, 10–20 mg tissue was added to 600 µl lysis buffer (10 mM Tris-HCl (pH 8.0); 1 mM EDTA (pH 8.0); 0.1% SDS) on ice before 60 µg PK (Promega) were added. After overnight incubation at 55 °C, the samples were cooled to room temperature before the addition of 12 µg DNase-free RNase (Qiagen) and a further incubation for 30 min at 37 °C. After cooling the samples to room temperature, 200 µl potassium acetate solution (60 ml 5 M KOAc, 11.5 ml glacial acetic acid, and 28.5 ml H₂O) was added, and the samples vigorously mixed and centrifuged for 3 min at 4 °C at a maximum speed. Genomic DNA contained in the supernatant was recovered by isopropanol precipitation, washed with 70% ethanol, and resuspended in 100 µl TE buffer. The resulting fragments for all three genes were cloned into a pCR4-TOPO vector (Invitrogen) and cycle sequenced as described previously. Due to the presence of large introns in the *20β-hsd* sequence, this gene was amplified in two overlapping fragments. 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 and detailed amplification procedures are available from the authors on request.

Quantitative real-time PCR

Primers and TaqMan fluorogenic probes specific for Atlantic cod *20β-hsd*, *cyp19a1*, and for the endogenous control *ef1a* were designed with Primer express software (Applied Biosystems), according to the manufacturer's guidelines. Probes for all three transcripts span exon-exon boundaries in the mRNA sequence to prevent genomic DNA amplification. The primers complementary to two different but consecutive exons 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 presence of an intron in the genomic DNA were further evaluated. A previously established assay for 18S rRNA of Atlantic salmon (Olsvik *et al.* 2005) was adapted for the use in Atlantic cod by changing the forward primer at three positions, based on 18S rRNA sequences published in GenBank (accession numbers AF518205, U11437, and U76257). Primers were purchased from Invitrogen, and TaqMan MGB fluorogenic probes were purchased from Applied Biosystems (Table 1). Optimal primers and probe concentrations were then determined following Applied Biosystems guidelines, and serial dilutions of RNA and cDNA were tested to assess the quality of the real-time PCR. The PCR solution contained 900 nM primers, 200 nM TaqMan probe, 1× AmpliTaq Gold TaqMan Mix (Applied Biosystems) and 2 µl template in a 25 µl volume. The PCR profile was as follows: 50 °C for 2 min, 40 cycles at 95 °C for 15 s, and 60 °C for 1 min, followed by 72 °C for 10 min. Two or three PCR were performed for each sample and then averaged. Data were analyzed by Sequence Detector version 1.6.3 (Applied Biosystems).

RNA extraction and reverse transcription

Samples stored at –80 °C were cut into small pieces on dry ice before immediate transfer to cold Tri Reagent (Sigma-Aldrich). 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 isopropanol precipitation or by purifying the RNA through a column (RiboPure kit, Ambion/Applied Biosystems), and quantified by spectrophotometry. Ten micrograms of RNA were DNase treated (TurboDNA-free, Ambion/Applied Biosystems) in 50 µl volumes before the remaining RNA was again quantified by spectrophotometry. Random-primed cDNA was synthesized from exactly 500 ng DNase-treated

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

	Sequence (5'–3')
Name	
aceef1a_q1	GCC CCT CCA GGA CGT CTA C
aceef1a	6FAM-AGA TCG GCG GTA TTG-MGB
aceef1a_q2	ACG GCC CAC GGG TAC TGT
ac20b-hsd_q1	GGT CAA CAA TGC TGG GAT AGC
ac20b-hsd	6FAM-TTT AAA GAA GCA GAC ACA ACA-MGB
ac20b-hsd_q2	GCC TGC ACG GCA AAC G
accyp19a_q1	ACA ACA ACA AGT ACG GCA GCA T
accyp19a	6FAM-TTG CAC AGG GTC ATG G-MGB
accyp19a_q2	GTA GAG GAG CTG CTG AGG ATG AG
ac18S_q1	CCC TGT AAT TGG AAT GAG TGT ACT TT
ac18S	6FAM-CAC CAG ACT TGC CCT CC-MGB
ac18S_q2	ACG CTA TTG GAG CTG GAA TTA CC

RNA using a Reverse Transcription Core Kit (RT-RTCK-05, Eurogentec, Seraing, Belgium), and diluted 1:10 in nuclease-free H₂O before use in the quantitative real-time PCR.

Assessment of RNA quality

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. The chip also generates a quantitative measure of the amount of RNA loaded.

Results

CYP19A1 (P450 aromatase)

A nearly full-length mRNA sequence of Atlantic cod *cyp19a1* was determined by a RACE-based strategy. Initially, a central part of the mRNA was amplified from ovarian cDNAs by RT-PCR using degenerate primers (Gen *et al.* 2001). This allowed us to design specific 5'- and 3'-RACE primers and subsequently to amplify 5' and 3' ends of the *cyp19a1* mRNA. After compiling consensus sequences from the overlapping fragments, primers were designed to amplify the whole presumptive coding regions in one single piece. The inferred mRNA sequence for aromatase consisted of 1645 nucleotides, with a coding region of 1602 nucleotides and short 5' untranslated region (UTR) of only 31 nucleotides. This was consistent across six independent 5'-RACE-clones. The 3' UTR consisted of 12 unique nucleotides, followed by poly(C) tracts of various length and GT repeats of even higher variability between the different 3'-RACE-clones. Since the focus of this work was not primarily to identify the longest possible UTRs, we did not further investigate the length and sequence of neither the 3' nor the 5' UTR. The deduced amino acid sequence consists of 533 residues and contains all the features characteristic of aromatases (Fig. 1). Similarity scores with other fish aromatases range from 56.2 to 68.9%.

The sequence of the corresponding genomic locus amplified using the primers designed to amplify the complete coding region, had a total length of 3882 nucleotides, and revealed the presence of nine exons and eight introns. The canonical sequence characteristics of vertebrate introns were completely conserved (Fig. 2), and the intron lengths ranged from 145 nucleotides for intron 5 to 591 nucleotides for intron 8.

Based on the determined exon-exon boundaries, a TaqMan assay was developed for *cyp19a1*. In order to have an assay that is specific for the gonad form of aromatase, the near complete sequence of the *cyp19a2* gene that codes for the neuronal form of aromatase was

determined from brain tissue (data not shown). Comparison of this sequence with the *cyp19a1* sequence revealed four mismatches in each primer and two mismatches in the probe, making it highly unlikely that this assay would lead to a signal with the brain form (*cyp19a2*) as a template. However, when screening various tissues for relative levels of expression, products were obtained readily also for the brain (Fig. 3). Sequencing of several of these products revealed perfect *cyp19a1* sequences (data not shown), demonstrating the specificity of this assay, and at the same time confirming that the gonad form of aromatase is also expressed to a certain extent in the brain of fish (Kwon *et al.* 2001, Trant *et al.* 2001, Choi *et al.* 2005). Other tissues that express aromatase are the testis, and interestingly we also detected expression in the male gill. Although the relative level of expression was low for this tissue, the positive signal was consistently obtained in repeated runs.

20 β -Hydroxysteroid dehydrogenase

During the attempts to determine the sequence of a central part of *cyp19a1*, two widely different sequences were amplified from ovarian samples collected at different time points during sexual maturation. Whereas one showed to be a central part of Atlantic cod *cyp19a1* as expected, the other one could be identified as a large part of 20 β -hsd. It can be speculated that this resulted from the presumably very high expression level of 20 β -hsd in the respective sample, together with the degeneracy of the primers. There were only seven mismatches in the 30 nt long forward primer when compared with the 20 β -hsd sequence, and only six in the 20 nt long reverse primer, in the latter all of them in the 5' part (data not shown). Nevertheless, this allowed us to design specific 5'- and 3'-RACE primers and to amplify the 5' and 3' ends of the 20 β -hsd mRNA. After compiling consensus sequences from the overlapping fragments, primers were designed to amplify the whole presumptive coding regions in one single piece.

The full-length mRNA sequence for 20 β -HSD had a length of 1232 nucleotides with an ORF of 825 nucleotides and 5' and 3' UTRs of 91 and 312 nucleotides respectively. In contrast to the *cyp19a1* gene, the complete sequence of the 3'-UTR could be determined without difficulty. The 3'-UTR ended with a poly(A) sequence, 21 nucleotides downstream of a perfect AATAAA polyadenylation signal, although another such signal was located 69 nucleotides further upstream. The deduced amino acid sequence of 275 residues showed the highest similarity with carbonyl reductase like 20 β -HSD sequences from other fish species, ranging from 57.1 to 79.6%. The characteristic features like the Rossmann-fold responsible for NADPH binding or the TyrXXXLys-motif crucial for catalytic activity were conserved in Atlantic cod, with the

	1.....10.....20.....30.....40.....50.....60.....70.....80	
G. morhua	--MDLFS-VCEWTVAPLRDLVLMVD-LGPRNATADTVA-----EVV-CVASRELLLLLLLLLLVAVTHRE-RATVPGP	67
D. labrax	--MDLIS-ACERAMTPVGLDTIVAD-LVSTSPNATAVG-----SPG-ISVATITLILLVCLLLVAVSHTD-KNTVPGP	67
O. mykiss	--MDLLSPVCGRVMAVVCCLDTVIADLLVSES RNATATR-----SEG-ISLATGSLLLLLCLLLAAWRHTD-NNSVPGP	69
D. rerio	MAGDLLQ-PCG--MKPVR LGEAVVDLLIQR AHNGT ERA-----QDNACGATATILLLLCLLLAIKRRHPKSHIPGP	70
I. punctatus	MAAHVFP-MCERTRKPVHFSETVMEILLREARNGTDPR-----YENPRGIT-LLLLLCLVLLLTVWRHEKKCSIPGP	71
A. japonica	-----MKHLEEIVMEALMPASRNATQTAGRVVSGATAALLSGATAALLLLCALLAAWSRSD-KSSVPGP	64
Transmembrane helix		
	90.....100.....110.....120.....130.....140.....150.....160	
G. morhua	YYCLGVGPLLCYLRFIWTGIGTASNYYNNKYGSMARVWINGEETLILSSSSTVRHVLRS AQYTSRFGSKQGLGCLGMDGR	147
D. labrax	SFCLGLGPLLSYLRFIWTGIGTASNYYNNKYGDIVRVWINGEETLILSRASVHVHLKNGHYTSRFGSKQGLSCMGMYER	147
O. mykiss	FFCLGVGPLLSYLRFIWTGIGTASNYYNSKYGDIVRVWINGEETFILSSSSAVHHVL RQGRYTSRFGSKQGLSCIGMDER	149
D. rerio	SFFPGLGPVVS YCRFIWSGIGTASNYYNSKYGDIVRVWINGEETLILSRSSAVYHVLRKSLYTSRFGSKLGLQCIGMHEQ	150
I. punctatus	SFFCLGLGPLMSYCRFIWMGIGTASNYYNEKYGDMVRVWISGEETLVLSRPSAVYHVLRKHSQYTSRFGSKLGLQCIGMHEQ	151
A. japonica	PFFYMIGIPLLSYFRFIWTGIGTASNYYNERGYDVRVWINGEETIILSRSSAVYQVLRKPQYTSRFGSKQGLRCIGMHQR	144
	170.....180.....190.....200.....210.....220.....230.....240	
G. morhua	GIIFNNDVALWKKTRSYFARALTGPGVLQTV DCTASTQSHLDSLADHPSSGPGEDLRQVDVLSVLRGIVDVSNTLFL	227
D. labrax	GIIFNNNVTLWKQIRNYFSKALTGPGLQQTVEVCVSSSTQTHLDDLDKLD-----NVDVLSLLRCTVVDISNRLFL	217
O. mykiss	GIIFNSNMALWKKTRTYFAKALTGPGLQRTVDVCVSSSTQTHLDALQGP DGLMG-----GQVDVLSLLRCTVVDISNRLFL	224
D. rerio	GIIFNSNVALWKKVRAFYAKALTGPGLQRTMEICTTSTNSHLDDLSQLTDAQG-----QLDILNLLRCIVDVSNRLFL	224
I. punctatus	GIIFNSNVLWRKVRTYFAKALTGPGLQRTLEICTMSTNTHLDGLSRLTDAQG-----HVDVLNLLRCIVDVSNRLFL	225
A. japonica	GIIFNNNIELWKKVRTYFAKALTGPGLQRTVATCVASTD SHLDQLEELTDL SG-----QVDILNLLRCTIVDISNQMFL	218
	250.....260.....270.....280.....290.....300.....310.....320	
G. morhua	GVPVDEKDLLAKILKYFDTWQTVLIKPDLYFKLDWIHRRQKDA AQELQDAIEVLIDLKRRELQ EADKLD RINF TADLIFA	307
D. labrax	GVPVNEKELLLKIQKYFDTWQTVLIKPDLYFKFDWIHQ RHKTA AQELQDAIESLVEQKRRDMEQADKLD- INF TADLIFA	296
O. mykiss	GVPLNEKELLQKI QKYFDTWQTVLIKPDVYFKLDWIHEKHRRAAQ ELED AIESLVDQKRRLQ EADKLDHINF TADLIFA	304
D. rerio	GVPLNEHDLLQKIHKYFDTWQTVLIKPDVYFRLDWLHRKHKRD A QELQDAITALIEQKVQLAHA EKLDHLDFT AELIFA	304
I. punctatus	DVPLNEQNLLFKIHYFETWQTVLIKPDYFR LKWLHDKHRNAAQELHDAIEDLIEQKRT ELQQA EKLDNLNFT EELIFA	305
A. japonica	RVPLNEKELLVKIQKYFEAWQTVLIRPDFLKF FEWYKEHKEAAHELHEAMEILVEKKRKAL EEA EKLD DAD FATDLIFA	298
	330.....340.....350.....360.....370.....380.....390.....400	
G. morhua	QSHGELSADHVRQCVLEMVIAAPDTLSVSLF MLLLLKQHPEVELRI L TEIDSVIGEGLRNE DLPQLCVME NFINESLR	387
D. labrax	QNRVGETAENVRQCVLEMVIAAPDTLSL FFM LLLLKQNP DVELQLLQEIDTVVGERQLQNGD LQRQLVLESFVNECLR	376
O. mykiss	QSHGELSAENVRQCVLEMVIAAPDTLSISL FFM LLLLKQNP DVELQLLEEIDTAIGDRELHNSDLQNL RVLESF FINE SLR	384
D. rerio	QSHGELSAENVRQCVLEMVIAAPDTLSISL FFM LLLLKQNP DVELKILQE MDSVLAGQSLQSHLSKLQILESF FINE SLR	384
I. punctatus	QSHGELTAENVRQCVLEMVIAAPDTLSISV FFM LLLLKQNAEVERRILTEIHTVLGDT ELQSHLSQLHVLECF FINE ALR	385
A. japonica	QNHGELSAENVQQCILEMIIAAPTLSISL FFM LMLLKQNP E VEQEILKELDTVIGDKKAENSNLHLIIMESF FINE SLR	378
I-helix		
	410.....420.....430.....440.....450.....460.....470.....480	
G. morhua	FHPVVDFTMRRLSDDVIDGYRVPKGTN IILNVRGMRHRETFY PKPNEFSLDNFEKSVPSRYFQFFGSGPRSCV GKHIA MV	467
D. labrax	FHPVVDFTMRRLSDDI IDGYRVPKGTN IILN TGMHMRTEFFFLKPN EFNLDNF KKNPPRRYFQFFGSGPRACV GKHIA MV	456
O. mykiss	FHPVVDFTMRRLSDDVISGYRVPKGTN IILNMGMRHRSEFFFLKPN EFSLDNFEKNIPNRFFQFFGSGPRSCV GKHIA MV	464
D. rerio	FHPVVDFTMRRLDDVDIEGYNVKGTN IILNVRGMRHSEFFSKPNQFSLDNFHKNPVSRFFQFFGSGPRSCV GKHIA MV	464
I. punctatus	FHPVVDFSYRRALDDVDIEGFRVPRGTN IILNVRGMRHSEFYPKPADFSLDNFNKVPVSRFFQFFGSGPRSCV GKHIA MV	465
A. japonica	YHPVVDFTMRKSLDDVDIEGYKVKGTN IILNVRGMHKEFFSKPN EFSLDNFEKTVNRRFFQFFGSGPRSCV GKHIS MV	458
Aromatase-specific		
	490.....500.....510.....520.....530.....540.....	
G. morhua	MMKSILVTLLSCYSLSPHEGLTLASMPQTNNLSQQPVESEQNPHYLAMTLTPRRRTATGSGYSAGAL	533
D. labrax	MMKSILVTLLSQYSVCPHKGLTLDCLPQTNNLSQQPVEHQQEA EHL SMRFLSRQRGSWKTL-----	518
O. mykiss	MMKSILVTLLSRYSVCPHEGLTLDCLPQTNNLSQQPVE--EEGEPHTMKFLPRHQARQS-----	523
D. rerio	MMKSILVALLSRFSVCPMKACTVENI PQTNNLSQQPVE--EPSSLSV-----	510
I. punctatus	MMKAVLLMVLRSFVCPESCTVENIAHTNDLSQQPVE--DKHTLSVRFI PRNTHTRNRKA----	525
A. japonica	MMKAILATLLSRYTMCPRDGR TLNNIRKTNNSQLLAE--KDSELTMMFTPRRRQ-----	512

Figure 1 ClustalX comparison of the deduced amino acid sequence of the *Gadus Morhua* P450 Aromatase (CYP19A1) open reading frame with a choice of teleost CYP19A1 sequences. Numbers to the right refer to the position of the amino acid. The predicted motifs of the transmembrane helix, the I-helix, the Ozol's peptide, the aromatase-specific region, and the heme-binding are shaded. Sequence information of Atlantic cod *cyp19a1* can be viewed in the NCBI GenBank database with accession number DQ402370.

exception of a conservative alanine to serine change at position 2 of the Rossmann-fold (data not shown).

The same primers were then applied on genomic DNA in order to determine the exact locations of introns.

Despite several attempts, the sequence of the corresponding genomic locus of the coding sequence of *20 β -hsd* could not be amplified in one piece, but rather had to be assembled from two overlapping fragments.

Gene	5' exon		Intron								3' exon		Intron length (nts)
	-2	-1	+1	+2	+3	+4		-3	-2	-1	+1	+2	
<i>20β-hsd</i>	A	T	G	T	A	G	...	T	A	G	T	G	515
	A	G	G	T	A	C	...	T	A	G	A	A	674
	A	G	G	T	A	C	...	C	A	G	G	T	92
	C	T	G	T	A	A	...	C	A	G	G	C	>616
	A	G	G	T	C	A	...	T	A	G	A	T	175
<i>cyp19a1</i>	A	G	G	T	C	A	...	C	A	G	G	C	233
	A	G	G	T	C	T	...	C	A	G	C	T	160
	A	G	G	T	G	C	...	T	A	G	C	T	170
	T	G	G	T	A	T	...	C	A	G	A	G	197
	G	C	G	T	G	G	...	C	A	G	T	C	145
	A	G	G	T	A	A	...	C	A	G	G	G	412
	A	G	G	T	A	A	...	A	A	G	G	C	365
	G	C	G	T	A	A	...	C	A	G	G	T	591
<i>eef1α</i>	A	G	G	T	A	A	...	C	A	G	A	T	306
	A	G	G	T	G	A	...	C	A	G	G	C	109
	A	G	G	T	G	A	...	T	A	G	A	T	109
	C	G	G	T	G	A	...	C	A	G	G	T	110
	A	G	G	T	G	A	...	C	A	G	G	T	107
	C	G	G	T	A	A	...	C	A	G	G	T	118
Consensus	A	G	<u>G</u>	<u>T</u>	A	A		C	<u>A</u>	<u>G</u>	G	T	
Frequency (%)	68	79	100	100	53	63		68	100	100	53	53	

Figure 2 Intron boundaries. Nucleotide sequences around the determined intron sites are shown for each of the three genes. Their frequency or degree of conservation for each position is indicated at the bottom. Sequence information of Atlantic cod *20β-hsd* and *eef1α* can be viewed in the NCBI GenBank database with accession numbers DQ402369 and DQ402371 respectively.

Nevertheless, the intron positions could be identified, and the length of the five introns ranged from 92 nucleotides for intron 3 to 674 nucleotides for intron 2 (Fig. 2). The only partially sequenced intron 4 was at least 600 nucleotides long, but might well be in the range of intron 2. The total length of sequence of the corresponding genomic locus of the coding sequence was slightly more than 3 kb, starting with the ATG and ending with the stop codon of the coding sequence.

Based on the determined exon–exon boundaries, an mRNA-specific TaqMan assay was developed also for *20β-hsd*. Screening of various tissues for relative levels of expression showed that *20β-hsd* is almost ubiquitously expressed, with the highest relative expression found in ovary and brain, and the lowest in intestine and muscle. There were no big differences between male and female tissues, with the exception of heart and gill, and of course the gonad, where the expression in the testis is very low when compared with that in the ovary.

Eukaryotic elongation factor 1α (EEF1α)

The mRNA sequence for EEF1α consisted of 1502 nucleotides including a 5'-UTR of 54 nucleotides and an incomplete 3'-UTR of 62 nucleotides. When this sequence was blasted against an expressed sequence tag library

generated in this laboratory, additional 237 nucleotides of the 3'-UTR were identified, making up a total of 1739 nucleotides for the mRNA. The ORF of 1386 nucleotide codes for a deduced protein of 461 amino acids, and the similarity scores to EEF1α protein sequences from other fish species was 86.1–91.2% (data not shown). The sequence for the corresponding genomic locus including six introns encompassed 2245 nucleotides, and the intron lengths ranged from 109 to 118 for introns 2 to 6, whereas the first intron was 306 nucleotides long.

RNA quality and seasonal changes in gene expression

When starting to extract tissue samples from different time points during an annual cycle, huge differences concerning RNA quality and quantity were observed (data not shown). Intact 28S and 18S rRNA peaks that are used by the software to assign an RNA integrity number (RIN value) to the samples were dwarfed by high amounts of one or several small RNA(s) in those samples collected in summer, when the gonads are still small and just start to grow. A slight change in the extraction method, from iso-propanol precipitation to column purification, enabled us to get more homogenous capillary electrophoresis profiles and more similar RNA amounts throughout a seasonal

Figure 3 Relative expression of *cyp19a1* (left side of the figure) and *20β-hsd* mRNA (right side of the figure) respectively in adult Atlantic cod tissue as determined by real-time quantitative RT-PCR. Designing assay with either probe or one of the primers spanning an exon–exon boundary ensures that the signal exclusively comes from mRNA. Expression in the ovary was set to 1. Where not stated otherwise, one organ from each gender was analyzed ($n=1$) except brain, where two female and one male brain samples were analyzed. Error bars are indicated where appropriate.

cycle. Ovarian samples collected from different time points during 1 year were analyzed for all four genes by q-RT-PCR, including the 18S rRNA that was chosen as an alternative standard or reference gene. Both genes showed some seasonal variation, with less fluctuation for 18S than for *eef1α* (Fig. 4). In order to gather more statistically relevant data, six samples from January to July 2005 were analyzed. The average C_t values for 18S were 10.50 in January and 11.35 in July, with an s.d. of 0.19 and 0.37 respectively. For the *eef1α* gene, the corresponding values were 21.73 (0.72) and 19.80 (0.81). This is in good agreement with the data obtained when running only one or two samples per month over a 9-month period. It indicates that both reference genes are sub-optimal, with 18S RNA being more stably expressed in this organ than *eef1α* mRNA. Aromatase and *20β-hsd* showed much higher variation between the data for January and those for July, with an upregulation of *cyp19a1* transcripts, and a downregulation of *20β-hsd* mRNA during the same period. At the same time, very low expression levels were observed in a testis sample from October that was run for comparison.

Figure 4 Absolute threshold cycle (C_t) values for the reference genes *eef1α* and 18S rRNA respectively to show the seasonal variation observed for samples taken from cod ovaries. The spawning season is indicated by a black bar.

Discussion

Aromatase is a key player in the enzymatic complex converting androgens (C19 steroids) to estrogens (C18 steroids). In mammals, only one *cyp19* gene exists, and tissue specificity and developmental expression patterns are generated by differential use of promoters. Teleosts, however, have two cytochrome P450 aromatase genes, probably generated by gene duplication during evolution (Chiang *et al.* 2001). The form primarily expressed in the ovary is designated *cyp19a1*, while the form predominantly expressed in the brain is termed *cyp19a2*. Since the expression level of at least its ovarian form mRNA correlates well with the enzymatic activity (Fukada *et al.* 1996), measurements of mRNA expression levels have been used as indicators of estrogen production capacity. For the first time, a complete *cyp19a1* sequence for Atlantic cod has been determined. The *cyp19a1* sequence assembled in this study contains all the typical features of an aromatase gene and was unambiguously assigned to the *cyp19a1* cluster of aromatase sequences (data not shown), distant from numerous fish *cyp19a2* sequences. However, the similarity to other fish *cyp19a1* sequences is surprisingly low (56.2–68.9% on the deduced amino acid level). Typically similarities between ovarian forms of teleost aromatases are at least 62%, often they reach more than 80% with the closest sequence. In the case of the two closely related tilapias (*Oreochromis niloticus* and *O. mossambicus*), it is even as high as 97%. Nevertheless, a partial *cyp19a1* sequence derived from Atlantic cod ovary has been deposited in GenBank (accession number AJ555405) and showed 99.7% identity to our sequence on the nucleotide level, demonstrating that this indeed represents the mRNA sequence for the ovarian form of aromatase in Atlantic cod. Together with the partial *cyp19a2* sequence also showing only low similarity scores to other fish *cyp19a2* sequences (65.1–80.1% on the deduced amino acid level), this indicates that the highly evolved gadoids have sequences very diverse from other fishes.

Since the ovarian form of aromatase is expressed to a certain extent also in the brain (and vice versa; Kwon *et al.* 2001, Trant *et al.* 2001, Choi *et al.* 2005), the nature of the positive signals obtained when running a screen of tissues including brain and gill was investigated. Surprisingly, low but consistently positive signals were obtained when running a male gill sample. While no expression of gonadal aromatase in gills of sea bass could be detected by a conventional RT-PCR (Blázquez & Pifferer 2004), expression of both *cyp19* genes was detected in the gills of rainbow trout (Dalla Valle *et al.* 2002) and orange-spotted grouper (Zhang *et al.* 2004) by Southern blotting of RT-PCR products. Low levels of *cyp19* mRNA were detected also in gills of southern flounder (Luckenbach *et al.* 2005), adding to the growing evidence that fish express aromatase locally in gills. However, it is not clear what function, if any,

aromatase might serve in this tissue or why it should be preferentially expressed in male cod. One might speculate that there is a need for certain levels of estradiol in this tissue, and in male fish this can only be produced locally by conversion from testosterone, mediated by locally expressed aromatase. In contrast, female fish have estradiol in the circulation, thereby contributing to the presence of certain levels of this steroid hormone even in the gills.

Sequencing of the products obtained by analyzing brain samples confirmed that the amplified target indeed was derived from the *cyp19a1* gene and ruled out possible cross-reactivity with the brain form. Comparison with the partial *cyp19a2* sequence also determined in this study (data not shown) revealed a conclusive number of differences. In addition, the primers and probes designed for this quantitative real-time PCR are highly unlikely to lead to a positive amplification signal for *cyp19a2*. We are therefore convinced that this assay represents a highly specific tool for the assessment of expression of the gonadal form of cytochrome P450 aromatase in Atlantic cod. To our knowledge, this is only the second time that such a highly specific and sensitive quantitative real-time PCR has been reported for a *cyp19* gene of a batch spawner. In Atlantic halibut (Matsuoka *et al.* 2006), expression of both *cyp19* isoforms was examined in juveniles in order to investigate sex differentiation. It remains to be seen if such an assay will be useful in studies on the regulation of vitellogenesis and final oocyte maturation in periodic spawners, such as cod and halibut.

The *20 β -hsd* sequence also showed only moderate similarity scores with other fish *20 β -hsd* sequences (57.1–79.6% on the deduced amino acid level), further confirming the evolutionary distant sequences of Atlantic cod. On the other hand, the features typical for carbonyl reductase like 20 β -HSD were conserved in Atlantic cod, strongly suggesting that the sequence determined in this study indeed represents the *20 β -hsd* gene of Atlantic cod. In agreement with the data obtained for rainbow trout (Guan *et al.* 1999) and Nile tilapia (Senthilkumaran *et al.* 2002), *20 β -hsd* was quite ubiquitously expressed. However, there seemed to be a sex-specific variation in *20 β -hsd* expression in heart and gills in Atlantic cod, in addition to the expected large differences in the gonads. Since none of the previous studies has looked at samples from both sexes, the significance of these findings needs to be further investigated. In conclusion, we are convinced that this assay is a similarly specific and sensitive tool for the assessment of *20 β -hsd* mRNA expression as the aromatase assay.

In search of a reference gene for quantification of mRNA expression by the $\Delta\Delta C_t$ method (Bustin 2000), we tested a number of genes either used in many studies (18S rRNA, β -actin) or suggested by previous experience in our institute (*eef1a*) (Frost & Nilsen 2003).

However, when carefully tested none of the genes fulfilled the stability criteria, all showed at least some seasonal variation in ovarian samples (Fig. 4 and data not shown), despite careful quantification of the RNA used for cDNA synthesis. One explanation for the large variation seen when running *eef1 α* and to a certain extent also 18S rRNA, is the asynchronous synthesis and storage of ribosomal components during oocyte growth (le Maire & Denis 1987, Thiry & Poncin 2005). The initial RNA extraction method using isopropanol precipitation of total RNA produced unexpected capillary electrophoresis profiles when ovarian samples from June to July were extracted. High peaks in the range of about 100 nucleotides completely dwarfed the normally predominant 18S and 28S rRNA peaks characteristic of intact total RNA. A similar phenomenon was observed in early studies of *Xenopus* oocytes where first 5S rRNA and to a certain extent also protein components of the ribosomes were synthesized before a switch occurred to 18S and 28S RNA production later in oogenesis (Scheer *et al.* 1976). A simple change in the extraction method from isopropanol precipitation to column purification removed the large peaks of presumably 5S rRNA and tRNA, but the seasonal trend in *eef1 α* remained (Fig. 4). It was therefore concluded that for future studies, when long-term series may be analyzed, instead of reference genes the input amount of RNA used for cDNA synthesis should be carefully standardized (Campbell *et al.* 2006).

In conclusion, we have determined the mRNA and genomic sequence of two key genes involved in oogenesis and final oocyte maturation. Sensitive mRNA-specific quantitative real-time PCR assays were established and evaluated for these genes as well as for *EEF1 α* . Testing of several candidate reference genes did not give satisfactory results, and careful standardization of input RNA was chosen for relative quantification of gene expression.

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