A cDNA for European sea bass (Dicentrarchus labrax) 11β-hydroxylase: Gene expression during the thermosensitive period and gonadogenesis

Silvia Socorro a,*, Rute S. Martins b, Laurence Deloffre b, Constantinos C. Mylonas c, Adelino V.M. Canario b,*

a Centro de Investigação em Ciências da Saúde, Faculdade de Ciências da Saúde, Universidade da Beira Interior, Av. Marquês d’Ava e Bolama, 6200 Covilhã, Portugal
b Center for Marine Sciences (CCMAR-CIMAR Associate Laboratory), Universidade do Algarve, Campus de Gambelas 8005-139 Faro, Portugal
c Hellenic Centre for Marine Research, Institute of Aquaculture, P.O. Box 2214, Iraklion, Crete 71003, Greece

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Abstract

Steroid P450 11β-hydroxylase, encoded by the CYP11B gene, is a key mitochondrial enzyme for the production of 11-oxygenated androgens, which have been shown to be potent masculinising steroids in several fish species. In this study we have isolated a CYP11B cDNA of 1903 base pairs from the testis of European sea bass (Dicentrarchus labrax) encoding a predicted protein of 552 amino acids. The amino acid identities to other vertebrate 11β-hydroxylase proteins ranged from 66% to 72% to other Wsh; 45% to amphibian and 35–39% to mammalian. Southern blot indicated that a single CYP11B gene is present. Northern blot analysis detected two transcripts in testis and head kidney, one of the same size of the isolated clone and the other of 3.9 kb. Reverse transcriptase-polymerase chain reaction showed abundant mRNA expression only in testis and head kidney, being residual in a range of other tissues. Expression of CYP11B and CYP19A (which encodes for ovarian aromatase) was detected from at least 4 days post-hatching and did not appear to be affected by rearing temperature (15 and 20 °C) during the first 60 days, a period in which high temperatures promote masculinisation in European sea bass. Throughout, gonadogenesis (60–300 dph), a highly dimorphic pattern of CYP11B expression was consistent with a role of this gene in testicular development.

1. Introduction

Gonadal sex differentiation in non-mammalian vertebrates is dependent upon the balance between androgens and estrogens at critical stages during development (Devlin and Nagahama, 2002; Ottinger and Abdelnabi, 1997). In teleosts, the most active testicular androgen is 11-ketotestosterone (11KT) (Borg, 1994; Devlin and Nagahama, 2002; Godwin et al., 2003) which is produced via a key hydroxylation effected by P450 11β-hydroxylase (CYP11B) (Kime, 1993). Among other functions, 11KT has been implicated in male sexual differentiation and gonadal development, as well as in the control of spermatogenesis (see reviews by Borg, 1994; Devlin and Nagahama, 2002). In teleosts, the same enzyme mediates corticosteroid 11β-hydroxylation in interrenal tissue (Colombo et al., 1972; Jiang et al., 1998). It is generally accepted that the main corticosteroid in fish is cortisol, which has both mineralocorticoid and glucocorticoid activity (Bern and Madsen, 1992). Aldosterone, the main mineralocorticoid in tetrapods, has only been detected sporadically in a few fish species at very low levels (Bern, 1967). While the CYP11B gene in fish encodes a protein possessing only 11β-hydroxylase activity (Jiang et al., 1998), in amphibians a single gene and a single protein account for both 11β-hydroxylase and aldosterone synthetase activity (Nonaka et al., 1995). Mammals appear

* Corresponding authors. Fax: +351 289800069. E-mail address: acanario@ualg.pt (A.V.M. Canario).
to have undergone one or more CYP11B gene duplications originating two or more CYP11B proteins of which one, CYP11B1, retained 11β-hydroxylase activity and the other, CYP11B2, retained aldosterone synthesizing activity (Bülow and Bernhardt, 2002; Lisurek and Bernhardt, 2004).

The European sea bass (Dicentrarchus labrax) is a gonochoristic species, whose chromosomal sex determination system (XX/YY or W/ZZ) is not clear (Blázquez et al., 1999). The critical period for androgen-inducible masculinisation lies between 96 and 126 days post-fertilization and administration of androgens during this period can result in all-male populations (Blázquez et al., 2001; Chatain et al., 1999). In addition, sex ratios can be modified by different incubation temperatures during the first three months of life, with generally higher temperatures (20–21°C) favouring masculinisation and lower temperatures (13–15°C) favouring more balanced sex ratios (Blázquez et al., 1998; Koumoundouros et al., 2002; Pavlidis et al., 2000; reviewed by Piferrer et al., 2005). However, some genotypes seem to be more susceptible to temperature (Sailant et al., 2002).

In the few studies available on steriodogenic enzyme expression in fish species with temperature-dependent sex differentiation (TSD), the mRNA expression of CYP19A (which encodes ovarian aromatase in teleost fishes) is suppressed by high temperatures in agreement with the masculinisation effects of high temperatures (D’Cotta et al., 1998; Koumoundouros et al., 2002; Pavlidis et al., 2000; reviewed by Piferrer et al., 2005). However, some genotypes seem to be more susceptible to temperature (Sailant et al., 2002).

This study, we report the cloning, phylogenetic analysis and tissue distribution of a CYP11B cDNA obtained from sea bass testis. We have also analysed the expression of CYP11B and CYP19A in fish grown at 15 and 20°C during the first 60 days post-hatching, covering the period in which sex differentiation in sea bass is influenced by temperature. Finally, since larger fish tend to be females, we have analysed the expression of CYP11B in populations of fish which were selected for large and small sizes from 60 to 300 days, covering the period of gonadal sex differentiation.

2. Materials and methods

2.1. Animals and experiments

Juvenile and adult European sea bass used to provide RNA for cloning and gene expression analysis in different tissues were obtained from Timar, Cultura de Águas (Livramento, Portugal) and maintained at the Ramalhete Experimental Station (University of Algarve, Faro, Portugal) prior to sampling in through-flow seawater tanks at 17±2°C under natural photoperiod. Animal care was in all cases in accordance with the ethical guidelines of the Animal Behaviour Society (ASAB, 2003), national and European legislation.

The fish utilized to investigate gene expression during the first 60 dph (days post-hatching) came from an experiment carried out in 2002 which was designed to study the effect of temperature on sex ratio. The experimental protocol was similar to that described by Mylonas et al. (2005, Northwestern Mediterranean Sea strain) with the difference that eggs and larvae were reared always at either 15 or 20°C until fish reached 18 mm.

Thereafter both groups were maintained at natural temperature (18–22°C) until sex determination was complete (300 dph) and sex was determined. Treatment resulted in 77% females in the 15°C group and 27% in the 20°C group. Samples for gene expression analysis were taken at 4, 18, 32, 46, and 60 dph. At 4 dph samples were 6 pools of 3 larvae per sample, at other times consisted of 6 individual larvae.

The fish to analyse gene expression during gonadal development in male and female dominant populations came from an experiment described in detail by Papadaki et al. (2005), carried out at the Institute of Aquaculture, Hellenic Center for Marine Research (Crete, Greece). Eggs were incubated at 17°C until hatching and from then on the larvae and juveniles were cultured at ambient temperatures throughout. The experiment consisted of grading the fish four times at ca. 50 days intervals, from 56 to 220 dph, each time retaining the 50% larger and the 50% smaller fish in the two groups that were formed after the first grading. Since European sea bass females tend to be larger than males, there was a progressive selection of females in the group with the larger fish and of males in the group with the smaller fish. Samples were taken from each grading but only from 150 days (second grading) it was possible to determine sex by histology.

2.2. Cloning of sea bass CYP11B and sequence analysis

Total RNA was extracted with TRI reagent (Sigma–Aldrich) according to the manufacturer’s instructions and reverse-transcribed (RT; 3–5 µg) in 40 µl at 37°C for 60 min, using Moloney murine leukemia virus (MMLV) reverse transcriptase (Invitrogen), oligo (dT)12–18 primer, 0.25 m M dNTPs, and 0.2 U of RNase inhibitor. A 900 bp cDNA fragment of the sea bass CYP11B (dlCYP11B) was obtained by polymerase chain reaction (PCR) with the degenerate primers P45011B and thermocycling conditions indicated in Table 1 in a final volume of 25 µl using 2 µl of synthesized cDNA, 1.25 U of Taq Polymerase (Promega, Madison, USA) and 25 pmol of each primer. The cDNA fragment was purified using the GFX PCR DNA and Gel Band Purification Kit (Amersham Pharmacia Biosciences, Lisbon, Portugal), cloned in pGEM-T Easy Vector (Promega) and sequenced. Identity was confirmed using BLASTX (Altschul et al., 1990) against the non-redundant nucleotide database in GenBank.

A complete coding sequence of dlCYP11B was obtained by screening at moderate stringency (in Church–Gilbert buffer at 55°C) with the radio-labeled cDNA probe (random labelling kit Rediprime II, Amersham Pharmacia Biosciences) a European sea bass testis cDNA library constructed in Lambda Zap using the UNIZAP vector (Stratagene). After screening 6 x 105 recombinants, four independent clones were autoexcised in pBlue-£er at 55°C) with the radio-

The putative dICYP11B cDNA was identified by searching the GenBank database with BLASTN and BLASTX (Altschul et al., 1990). Multiple sequence alignments of the translated European sea bass sequence were carried out with ClustalX (version 1.64b) and a maximum parsimony phylogenetic tree for CYP11B was obtained with PAUP* (version 4.0b, Swofford, 1998) using available sequences from the Uniprot database: CYP11A—-Ictalurus punctatus O73852, Onchorynchus mykiss Q07217, Brachydanio rerio Q8JH93, Poephila guttata Q6GK8, Gallus gallus O13254; CYP11B—Anguilla japonica (Jiang et al., 1996), Onchorynchus
Table 1
Primers and thermocycling conditions used in the PCR.

<table>
<thead>
<tr>
<th>Primers (5′–3′)</th>
<th>Thermocycles</th>
<th>Product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>P450&lt;sub&gt;11b&lt;/sub&gt; Sense</td>
<td>YGCNYTNGGNCCNATMTAYAGG</td>
<td>35 cycles: 95 °C 1 min, 56 °C 1 min, 72 °C 1 min</td>
</tr>
<tr>
<td>Antisense</td>
<td>GGRTTCCCKNGGC ARYTCAAANA</td>
<td></td>
</tr>
<tr>
<td>ro11b1 Sense</td>
<td>AAGAACCGTGAGGAGTGAGC</td>
<td>26 cycles: 94 °C 30 s, 58 °C 30 s, 72 °C 30 s</td>
</tr>
<tr>
<td>Antisense</td>
<td>TGGAGGGAGATGAGGAGAG</td>
<td></td>
</tr>
<tr>
<td>dlarom Sense</td>
<td>ACAGACACGGTGCGCACTGAC</td>
<td>22 cycles: 94 °C 1 min, 58 °C 1 min, 72 °C 1 min</td>
</tr>
<tr>
<td>Antisense</td>
<td>CTGAACCGAATGGCCTGGAAGT</td>
<td></td>
</tr>
<tr>
<td>18S Sense</td>
<td>TCAAGACGCGAAGTGGAGGG</td>
<td>18 Cycles: 94 °C 30 s, 59 °C 30 s, 72 °C 30 s</td>
</tr>
<tr>
<td>Antisense</td>
<td>GGACATCTAAGGGCATCACA</td>
<td></td>
</tr>
</tbody>
</table>

2.3. Sequence and phylogenetic analysis of dlCYP11B and dlCYP19A

RT-PCR was used to determine the expression of dlCYP11B and dlCYP19A relative to the 18S subunit of ribosomal RNA using the basic principle described by Nakayama et al. (1992). The linear range of the PCR reaction was determined by taking aliquots of the products every other cycle between 10 and 35 cycles. The products were run on agarose gel, visualized with ethidium bromide and the images stored using Image Master VDS (Amersham Pharmacia). The intensity of each band was determined by image analysis Image Master 1D Prime software version 3.00 (Amersham Pharmacia) and plotted against cycle number to determine the cycles in the linear range of amplification. Total RNA from larvae and male and female tissues of different ages was isolated and reverse transcribed as above and the cDNAs used for RT-PCR with the primers ro11B1 directed at dlCYP11B and dlarom directed at dlCYP19A (Accession No. AJ311177; Dalla Valle et al., 2002) and 18S (Table 1) in a reaction volume of 25 μl. The thermocycling conditions were optimised to obtain a single product in the linear range for the PCR reaction (Table 1). RT-PCR products were separated on agarose gel (0.7%), denatured (1.5 M NaCl/0.5 M NaOH) and neutralised (1.5 M NaCl/0.5 M Tris–Base, pH 8) and transferred onto nylon membranes (Hybond XL, Amersham Pharmacia) in 6× SSC solution. The membranes were hybridized with the respective cDNA probe, 161 bp dlCYP11B and 450 bp 18S, radiolabelled in a phosphorimager (GS-505 Molecular Imager System, Biorad) and results expressed as the ratio of amplified target over 18S rRNA. The ratios of dlCYP11B in relation to 18S RNA were log transformed and the effects of temperature or grading and age were analysed by two-way Analysis of Variance followed by the Holm–Sidak multiple comparison method at a level of significance of 5%.

3. Results

3.1. Sequence and phylogenetic analysis of dlCYP11B

The RT-PCR cDNA fragment obtained from interrenal tissue and the 4 clones obtained from screening the testis cDNA library had identical nucleotide sequences and highest similarity to the fish CYP11B sequences. The dlCYP11B sequence has been deposited in GenBank with Accession No. AF449173. The testis clones were 1903 bp long, containing an open-reading-frame of 552 amino acids, a 46 bp 5′-UTR and a 203 bp 3′-UTR, to which a poly (A)<sup>+</sup> tail was attached and a polyadenilation signal located 15 bp upstream.

The CYP11B amino acid sequence contained typical P450 conserved features as indicated in Fig. 1. Fish sequences possess three inserts of ca. 5–12 amino acids before the steroid binding region, between this region and
the oxygen binding region and between the aromatic region and the heme-binding region. The overall amino acid identities between dlCYP11B and other CYP11B sequences ranged between 64% and 79% for teleost \textit{W}sh, 45% for amphibian and 35–39% for mammalian. The phylogenetic analysis cluster dlCYP11B with the other \textit{W}sh sequences, separated from amphibian, birds and mammals (Fig. 2).

3.2. Gene copies and tissue distribution of mRNA transcripts

A single band was obtained from the hybridization of genomic DNA digest with the dlCYP11B probe (Fig. 3) indicating the presence of a single copy gene in the European sea bass genome. Analysis of dlCYP11B transcripts by Northern blot (Fig. 4) identified two transcripts of 2 and 3.9 kb, in testis and head kidney. In both tissues, the 2 kb transcript was the most abundant corresponding in size to the isolated cDNA. No signal was detected in ovary. Analysis of the expression pattern of dlCYP11B in different tissues (Fig. 5) indicated that in both males and females dlCYP11B was strongly expressed in the head kidney and also in testis in males. Very low levels were detected in other tissues for both sexes.

3.3. dlCYP11B and CYP19A expression during early larval development and the effect of temperature

There was neither a statistically significant interaction between age and temperature nor between the 15 and 20 °C developmental stages for both sexes.
in the mRNA expression of dlCYP11B (Fig. 6). However, the relative expression of dlCYP11B was significantly lower at 18 and 60 days post-hatching compared to the other time points. Although there was a significant interaction between age and temperature \((P=0.018)\), there was no significant difference between the mRNA expression levels of dlCYP19A at 15 and 20 °C \((P=0.23)\) (Fig. 7).

3.4. dlCYP11B expression during gonadogenesis

There was a significant interaction \((P<0.05)\) in mRNA expression of dlCYP11B between age and size indicating that difference in the level of expression between the two size-graded groups changed with the age of the fish (Fig. 8). In the larger size group, dominated by females, mean expression levels did not change significantly throughout the experiment. In the group of smaller fish there was a progressive increase in expression levels which from day 200
was significantly higher than 56 days and of fish of similar age in the larger size group.

4. Discussion

A full-length cDNA has been isolated from a European sea bass testis with an open reading frame encoding a protein with 552 amino acids sharing highest amino acid similarity to medaka, rainbow trout and eel CYP11B, than with amphibian and mammalian. A multiple sequence alignment of CYP11B spanning all vertebrates (Fig. 1) shows high conservation in P450 characteristic regions such as the steroid- and oxygen-binding, the Ozols', the aromatic and the heme-binding regions (Nonaka et al., 1995). Of the 128 residues conserved in all vertebrates, 42 are located in those regions, which in total, span 78 amino acids. The remaining
absolutely conserved residues are distributed along the molecule. The N-terminal 30–60 residues which correspond to a membrane spanning region (Nonaka et al., 1995) is not very well conserved.

Amino acids G288 and A320 of human CYP11B2, which are necessary for strong aldosterone synthase activity (Curnow et al., 1997; Mulatero et al., 1998), are simultaneously conserved in all CYP11B sequences known to have aldosterone synthase activity. However, the same amino acids are present in for example CYP11B3 (Ogishima et al., 1989) and in Japanese eel (Jiang et al., 1998) but neither possess aldosterone synthesizing capacity (Jiang et al., 1998). In the absence of a better explanation, the lack of aldosterone synthesizing activity in teleost fish CYP11B
may be contributed by the presence of the three short amino acid inserts which are likely to modify the tertiary structure of the molecule.

The presence of aldosterone in teleost fish has been a controversial issue. There are very few studies reporting the presence of aldosterone and only at residual levels in fish plasma or as a product of interrenal biosynthesis (Blair-West et al., 1977; Reינking, 1983). As such, it has been assumed that the majority of teleost fish do not synthesise aldosterone, and that in the absence of this steroid, cortisol plays a double role in the metabolism of carbohydrates and in salt/water balance (Bern and Madsen, 1992; Wendelaar Bonga, 1997). However, a mineralocorticoid receptor with highest affinity for both cortisol and aldosterone has been identified in teleost fish (Colombe et al., 2000; Greenwood et al., 2003). More recently, it has been reported that 11-deoxycorticosterone (DOC) binds and activates two mineralocorticoid isoforms with equipotency to aldosterone (Sturm et al., 2005). However, whether DOC is a mineralocorticoid in fish needs to be demonstrated.

The number and size of the transcripts found in testis and in the head kidney are similar as indicated by Northern blot. The dICYP11B cDNA most likely corresponds to the smaller 2 kb mRNA transcript found in both tissues. There was, however, a 3.9 kb mRNA transcript present in testis and in head kidney. It is possible that this second transcript differs in the size of 3′-UTR due to the use of alternative polyadenilation signals as exemplified by many other eukaryotic genes (e.g. Boyd et al., 1995). Additional hybridization studies with partial probes will be necessary to confirm this hypothesis.

In addition to its role in corticosteroid production, 11β-hydroxylase is essential for o xoandrogens production in fish gonads, in particular 11KT, which is implicated in the promotion of primary and secondary sex characters and sexual and agonistic behaviour (Borg, 1994; Devlin and Nagahama, 2002; Oliveira et al., 2001). For example, 11KT can be detected in the developing embryo and larvae (Yeo et al., 1996) and levels increase when the male European sea bass gonad differentiates (Papadaki et al., 2005). Furthermore, 11KT promotes testicular differentiation in undifferentiated fish (Yamamoto, 1969) and in gonadectomized female goldfish (Kobayashi et al., 1991) and germ cell differentiation in vitro in Japanese eel (Miura et al., 1991).

A specific role for CYP11B in the testis has been also revealed by the RT-PCR analysis, which showed that in two-year-old European sea bass it was expressed in testis but not in ovary. Nevertheless it was expressed in the head kidney of both sexes. The pattern of dICYP11B expression was similar to that described for the Japanese eel (Jiang et al., 1998) and the rainbow trout (Kusakabe et al., 2002; Liu et al., 2000), and was coincident with the androgen and glucocorticoid production sites. The absence of expression in the ovary indicates lack or low production of 11β-oxygenated androgens in the European sea bass ovary. Consistent with this is the observation, during the sex differentiating period (after 90 days), the group of larger fish (female biased population) presents residual levels of CYP11B mRNA expression, while in the group of smaller fish (male biased population) it is detected at increasing levels. Nonetheless, dICYP11B expression levels may have been underestimated in younger larvae since it was assayed from whole larvae at 56 days, body trunks at intermediate stages and from the gonad at 300 days. During sex differentiation CYP11B mRNA also expresses at two orders of magnitude higher level in male than in female rainbow trout gonads (Liu et al., 2000). Thus, the highly dimorphic pattern of expression obtained in the present study during and after gonadal differentiation, suggests that CYP11B is acting through the male primary sex differentiation pathway, leading to a testis, probably through the production of masculinizing 11-oxygenated androgens.

In European sea bass, CYP11B mRNA is detected as early as day 4 post hatching, before the sex determining period (Blázquez et al., 2001) and before the development of the gonadal tissue, suggesting that steroid, most likely corticosteroid, production in the interrenal begins very early in development (Hwang et al., 1992; Stouthart et al., 1998). Early expression of CYP11B has also been reported for rainbow trout (Liu et al., 2000) and the Nile tilapia (D’Cotta et al., 2001b). Although in our study more males (69%) were obtained in the group maintained at 20°C, we could not find significant differences in CYP11B expression between the two groups. The oscillations of CYP11B expression at different points in time and among individuals may indicate a possible role during larval development, but not necessarily related to reproductive structures. This is unlike the observations in Nile tilapia, where there was a marked upregulation of CYP11B in genetic males reared at masculinising temperatures (35°C), compared to males reared at natural temperature (27°C), indicating an influence of temperature on CYP11B and masculinisation (D’Cotta et al., 2001b). Although P450-aromatase activity and mRNA expression is downregulated by emasculating temperatures in Nile tilapia (D’Cotta et al., 2001a), we could not find a differential pattern of expression related to temperature in European sea bass at the sampling points analysed, despite CYP19A mRNA being detected from at least 4 days post-hatching. Thus, it appears that neither CYP11B nor CYP19A is temperature sensitive within the masculinising window for European sea bass. This indicates that the temperature effects on sex ratios during the first 60 days post-hatching in European sea bass (Koumoundouros et al., 2002) is not mediated by aromatase or 11β-hydroxylase, but by some other yet unknown factor. Consistent with this hypothesis, in some reptiles with temperature-dependent sex differentiation, CYP19 expression is not related to temperature but with ovarian differentiation, thus implicating upstream genes (Gabriel et al., 2001; Murdoch and Wibbels, 2003). This possibility cannot be ruled out in European sea bass and it is even possible that temperature acts at more than one point in the sex differentiation pathway. On the basis of known expression patterns in other fish species, and by analogy to their role in higher
vertebrates, possible upstream candidates genes could be steroidogenic factor 1 (Nr5a1) (von Hofsten et al., 2001) or Nr0b1 (formerly dax1) (Wang et al., 2002), both of which are involved in the steroidogenic pathway (Lalli and Sassone-Corsi, 2003). This possibility is being investigated.

In summary, we report the cloning of a cDNA encoding for 11β-hydroxylase, a critical enzyme for the production of corticosteroids and 11-oxoandrogens, with a mRNA expression pattern consistent with its known roles in reproduction, stress and sex differentiation. The expression data also suggests that the masculinising effect of higher incubation temperature during early larval development may not be mediated by regulation of the expression of either CYP11B or CYP19A but by upstream genes before sex differentiation starts.

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References


