

***DAX1* a sex determining gene in fish?**

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DAX1, a sex determining gene in fish?

Abstract

Sex determination is an essential process for the survival and evolution of vertebrate species. Although most vertebrates rely on a classical system of male heterogamety (males are XY whereas females are XX) or variations thereof, different fish species do not rely exclusively on these classical chromosomal systems, and instead, display a diverse array of sex determining mechanisms that include different forms of hermaphroditism and environmental sex determination. Thus, no simple genetic sex determining system can be generalized for fish. However, different studies have shown that most of the genes that are involved within the genetic sex determining cascade in vertebrate species that use chromosomal systems are also found in fish species that appear not to have heterogametic sex chromosomes, suggesting that they may have conserved functions despite the sex determining system in use.

One of the genes that have been shown to be determinant for mammalian sex determination is the *DAX1* gene. Indeed, mutations in human *DAX1* and *DAX1* mouse knockout models have resulted in genetic male sex reversal. Interestingly, this gene has been isolated in two different fish species and, due to its high conservation, led us to hypothesize that it may also have retained similar functions in fish. In order to test this hypothesis, we have decided to work with the European sea bass, which is a fish species that lack heterogametic sex chromosomes, but possess a sex determining mechanism that is strongly influenced by temperature (TSD). Thus, we have proceeded to the isolation of the full length sequence and gene structure of *DAX1* in the European sea bass (*sbDAX1*). In the process, we have also identified a duplicate *DAX* gene (named *sbDAX2*), and another member of the *DAX1* nuclear receptor family called *SHP* (*sbSHP*). The three nuclear receptors presented a conserved gene structure and putative protein domains as found in mammalian homolog genes.

The tissue distribution of the three nuclear receptor members revealed a dimorphic pattern of expression within the hypothalamus-pituitary-gonadal (HPG) axis of male and female fish, suggesting that they may be involved in reproduction. In addition, during the sex determining period, larvae grown at either feminizing (15°C) or masculinizing (21°C) temperatures also presented a dimorphic pattern of expression of all three receptors, since larvae grown at low temperature showed repressed gene expression, while at higher temperature they were significantly upregulated. In addition, mRNA expression of other candidate sex determining genes, *SOX9.1*, *SOX9.2* and *SF1*, was also similar to that obtained for the *DAX* duplicate genes and *SHP*. Thus, we have presented data

showing that during the temperature sensitive sex determining period, feminizing or masculinizing temperature regimes affect differently the transcription of different putative sex determining genes, namely of *DAX1* and *DAX2*.

We have also isolated the sea bass *DAX1* promoter sequence and compared it with that of other vertebrates. The promoter architecture of vertebrate *DAX* promoters was remarkably conserved from fish to mammalian species, and we have identified different promoter frameworks that are conserved in fish and mammals. In addition, we have also identified other gene promoters annotated in the Genbank database that also shared one or more of these frameworks, enabling us to establish a putative role of *DAX1*, together with the CaM signalling pathway, in the regulation of steroid production within the gonads. To test this hypothesis, we have demonstrated that in male gonads, steroidogenic capacity is highly dependent on the availability of calmodulin within the cells, since inhibition of this protein with an antagonist (W7) blocked steroid production in the gonads of male tilapia, *in vitro* and *in vivo*. In addition, we have also studied the *DAX1* and *DAX2* mRNA expression in testis of tilapias treated with W7, and found that downregulation of the CaM signalling pathway leads to the upregulation of *DAX1* and *DAX2* transcription levels.

In conclusion, although we did not obtain any conclusive data supporting a role for fish *DAX1* and *DAX2* genes in either male specific or female specific sex determination, we did find a link between temperature and *DAX1* and 2 transcription levels during the TSD supporting the view of a role of these genes within the early gene cascade involved in TSD. In addition, we have also shown that Ca^{2+} -CaM signalling pathway is not only involved in the modulation of steroid production within male fish gonads as it is also involved directly or indirectly in the modulation of both *DAX1* and *DAX2* gene transcription within the gonads.

DAX1, un gène du déterminisme génétique du sexe chez les poissons ?

Résumé

La détermination du sexe est un processus essentiel à la survie et l'évolution chez les vertébrés. Bien que la détermination du sexe chez la plupart des vertébrés soit basée principalement sur l'hétérogamétie (mâles XY et femelle XX), les différentes espèces de poissons ne présentent pas forcément un tel système de chromosomes sexuels, mais plutôt divers types de déterminismes sexuels tels que différentes formes d'hermaphrodismes et de déterminismes épigénétiques. Il n'existe donc pas un type général de déterminisme génétique du sexe chez les poissons. Cependant, différentes études ont montré que la plupart des gènes impliqués dans le déterminisme génétique du sexe chez les autres vertébrés, ce dernier étant basé sur un système de chromosomes sexuels, sont aussi présents chez les poissons. Ceci suggère que ces gènes pourraient néanmoins avoir une fonction similaire dans le déterminisme du sexe chez les poissons malgré l'absence d'hétérochromosomes.

DAX1 est un gène essentiel pour la détermination du sexe chez les mammifères. En effet, des mutations du gène *DAX1* chez l'homme et sur des modèles de souris «knockout» aboutissent à une réversion sexuelle mâle. *DAX1* a été isolé chez deux espèces de poissons, et étant donné son taux de conservation important nous avons formulé l'hypothèse que ce gène pourrait avoir chez les poissons une fonction similaire à celle qui est connue chez les mammifères. Nous avons choisi le loup ou bar, *Dicentrarchus labrax*, comme modèle d'étude, un poisson chez qui il n'existe pas d'hétérochromosomes. Chez cette espèce, le déterminisme du sexe est fortement dépendant de la température (TSD). Ainsi, nous avons isolé la séquence complète et déterminé la structure du gène *DAX1* chez *D. labrax*. Faisant cela, nous avons également identifié chez notre modèle, un duplicata de ce gène ou *sbDAX2*, et un autre gène faisant partie de la famille des récepteurs nucléaires *DAX1*, appelé *SHP* (*sbSHP*). Ces trois gènes, *sbDAX1*, *sbDAX2* et *sbSHP*, appartenant à la famille des récepteurs nucléaires, présentent une structure conservée et des domaines protéiques putatifs similaires à ceux des gènes mammaliens homologues.

L'étude de l'expression de ces trois gènes dans les différents tissus de *D. labrax* révèle une expression di-morphique au niveau de l'axe hypothalamus-hypophyse-gonadique chez les mâles et les femelles, suggérant un rôle de ces gènes dans la reproduction. L'expression de ces trois récepteurs nucléaires présente également un

modèle di-morphique au cours de la période de détermination sexuelle puisque l'expression des gènes est réprimée à une température basse féminisante (15°C), alors qu'elle est significativement augmentée à une température élevée masculinisante (21°C). Une modèle d'expression similaire à celui observé pour les gènes dupliqué *DAX* et le gène *SHP* a également été observé chez *D. labrax* pour d'autres gènes candidats à la détermination sexuelle, *SOX9.1*, *SOX9.2* et *SF1*. Nos résultats montrent donc que pendant la période critique de la détermination du sexe température-dépendante, les températures féminisantes ou masculinisantes agissent différemment sur la transcription des gènes que nous pensons être impliqués dans la détermination sexuelle chez *D. labrax*, c'est-à-dire *DAX1* et *DAX2*.

Le promoteur du gène *DAX1* a été isolé chez *D. labrax* et sa séquence comparée à celles d'autres vertébrés. La structure du promoteur des gènes *DAX* semble remarquablement conservée des poissons jusqu'au mammifères et nous avons identifié différents portions de promoteurs identiques chez les poissons et les mammifères. En utilisant la base de données Genbank, nous avons aussi identifié d'autres gènes comportant une ou plusieurs de ces portions de promoteurs, ceci nous permettant de conclure à un possible rôle de *DAX1*, ensemble avec la voie de signalisation calmoduline (CaM), dans la régulation de la production de stéroïdes par les gonades. Pour tester cette hypothèse, nous avons démontré que dans la gonade mâle, la capacité stéroïdienne est hautement dépendante de la présence de CaM dans les cellules puisque l'inhibition de cette protéine par l'antagoniste W7 bloque la production de stéroïdes dans la gonade mâle du tilapia, et ceci *in vitro* et *in vivo*. Nous avons également étudié l'expression des ARNm de *DAX1* et *DAX2* dans le testicule du tilapia traité avec W7 et trouvé que l'inhibition de la voie de signalisation CaM entraîne une augmentation de la transcription de *DAX1* et *DAX2*.

En conclusion, malgré l'absence de données permettant de conclure avec certitude au rôle des gènes *DAX1* et *DAX2* dans le déterminisme sexuel mâle ou femelle, nous avons trouvé un lien entre la température et les niveaux de transcription de *DAX1* et *DAX2* au cours de la TSD. Ceci conforte l'opinion selon laquelle ces gènes sont liés à la cascade de gènes activés précocement et impliqués dans le TSD. Nous avons de plus montré que la voie de transmission du signal Ca^{2+} -CaM est non seulement impliquée dans la régulation de production de stéroïdes dans la gonade mâle du poisson mais est également impliquée directement ou indirectement dans la régulation de la transcription des gènes *DAX1* et *DAX2* dans la gonade.

DAX1, um gene que determina o sexo em peixes?

Sumário

O processo de determinação sexual é essencial à sobrevivência e evolução de todas as espécies de vertebrados. Embora a determinação do sexo na maioria de espécies esteja associada à presença de cromossomas sexuais (e.x., na maioria dos mamíferos, os machos possuem XY e as fêmeas XX), diversas espécies de peixes possuem sistemas de determinação sexual que não estão directamente associados à presença de cromossomas sexuais, uma vez que neste grupo de vertebrados existem diferentes espécies que são hermafroditas ou que utilizam mecanismos dependentes da temperatura ambiental. Assim sendo, não é possível criar um modelo genérico que possa explicar o processo de determinação sexual neste grupo de vertebrados. No entanto, estudos efectuados em peixes mostram que, a maioria dos genes envolvidos no processo de determinação sexual em mamíferos que utilizam cromossomas sexuais, estão também presentes em espécies de peixe que não possuem cromossomas sexuais. Estes estudos sugerem que os genes envolvidos no processo de determinação sexual em vertebrados são bastante conservados, e que, independentemente da presença ou ausência de cromossomas sexuais, as funções destes genes nas diversas espécies, deverão ser bastante conservadas.

Um dos genes que está envolvido no processo de determinação sexual em mamíferos é o *DAX1*. A ocorrência de mutações neste gene em humanos ou a sua ablação do genoma de ratos resulta frequentemente na inversão sexual de machos genéticos. Recentemente, foram identificados genes homólogos do *DAX1* em duas espécies de peixe. Dada a elevada conservação ao nível da sequência, nós colocámos como hipótese que provavelmente também deveriam apresentar funções conservadas no processo de determinação sexual dos peixes, independentemente da ausência de cromossomas sexuais. Para testar esta hipótese, decidimos trabalhar com o robalo, *Dicentrarchus labrax*, que é uma espécie de peixe cujo processo de determinação sexual pode ser manipulado através da utilização de diferentes regimes de temperatura nas fases iniciais de desenvolvimento larvar. Assim, começámos por isolar o gene homólogo do *DAX1* (designado neste trabalho *sbDAX1*), caracterizar a estrutura do gene e identificar a sequência complementar do ARN mensageiro que é expressa nesta espécie. Durante este processo, identificámos ainda um gene resultante da duplicação genómica do *DAX1* nesta espécie (designado *sbDAX2*) e de um outro membro da família dos receptores nucleares orfãos à qual o *DAX1* pertence, *SHP* (designado neste trabalho

sb*SHP*). A identificação destes três genes permitiu-nos verificar que em peixes, os três genes possuem uma elevada conservação ao nível da estrutura dos genes, assim como da sequência e principais domínios das proteínas que codificam e, que a presença de uma duplicação do gene *DAX1* é específica nos genomas de peixes.

O estudo da expressão destes três receptores orfãos nucleares em diferentes tecidos mostrou que todos eles apresentam um padrão dimórfico entre machos e fêmeas, nos tecidos pertencentes ao eixo do hipotálamo-pituitária-gónadas (eixo HPG), o que sugere um provável papel destes genes no processo da reprodução em peixes. Durante o período no qual existe maior sensibilidade para manipulações dos sexos pela temperatura (nos 60 dias iniciais após eclosão), a expressão dos três receptores nucleares também apresentaram um padrão dimórfico em larvas cultivadas a temperaturas que favorecem o desenvolvimento de fêmeas (15°C) ou de machos (21°C). Ou seja, larvas cultivadas a baixas temperaturas apresentam níveis baixos de expressão de sb*DAX1*, sb*DAX2* e sb*SHP*, enquanto que a altas temperaturas os níveis de expressão destes receptores se apresentaram significativamente estimulados. O estudo da expressão de outros genes que se encontram igualmente envolvidos no processo de determinação sexual em mamíferos, *SOX9* e *SF1*, nestas larvas mostrou ainda que todos eles apresentam também o mesmo padrão de expressão dimórfico. Assim, os dados presentes neste trabalho mostram que durante o período de maior sensibilidade à temperatura, a utilização de diferentes regimes de temperatura no cultivo de larvas de robalo afecta significativamente a transcrição de diferentes genes homólogos aos envolvidos no processo de determinação sexual em mamíferos, nomeadamente do *DAX1* e *DAX2*.

No decurso deste trabalho isolámos ainda a sequência do promotor do *DAX1* do robalo e comparámo-la com as sequências dos promotores de outros vertebrados. A estrutura e arquitectura do promotor do *DAX1* dos peixes apresenta uma elevada conservação quando comparados aos dos mamíferos. Em todos eles, nós identificámos blocos de conservação que se encontram também em diversos promotores de genes anotados na base de dados do Genbank, o que nos permitiu inferir um possível envolvimento do *DAX1* de peixes em diversos processos fisiológicos, nomeadamente na sinalização intracelular via Ca^{2+} -Calmodulina (Ca^{2+} -CaM) na produção de esteróides sexuais nas gónadas. Para testar esta hipótese começámos por demonstrar que, nos testículos de tilápia Mossambicana, a produção basal de esteróides sexuais é fortemente regulada via Ca^{2+} -CaM. Assim, demonstrámos que a administração de um inibidor de CaM (W7) leva ao bloqueio da produção de testosterona e 11-cetotestosterona, *in vitro* e *in vivo*. De seguida caracterizámos o impacto da administração *in vivo* deste inibidor na transcrição do *DAX1* e *DAX2* nas gónadas de tilápia e demonstrámos que a inibição

desta via de sinalização intracelular leva ao aumento significativo dos níveis de RNA mensageiro destes receptores.

Em conclusão, embora não tenhamos obtido dados conclusivos demonstrando que nos peixes o *DAX1* e *DAX2* são genes de determinação sexual específicos de fêmea ou de macho, nós demonstrámos que a utilização de diferentes regimes de temperatura para manipulação da determinação sexual afecta os níveis de transcrição destes genes, o que sugere que muito provavelmente estarão envolvidos na determinação genética do sexo em peixes. Neste trabalho demonstrámos ainda que a produção de androgénios nos machos é regulada via sinalização intracelular de Ca^{2+} -CaM e que a presença de calmodulina disponível nas células estimula a produção de esteróides, enquanto que limita directa ou indirectamente a transcrição de *DAX1* e *DAX2*, sugerindo um papel repressor destes receptores no processo da esteroidogenese.

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CHAPTER 1

General introduction

1.1 The quest for a sex determining gene in fish

Sex determination is an integral part of reproduction and an essential process for the survival and evolution of vertebrate species. Most vertebrates rely on a classical system of male heterogamety (males are XY whereas females are XX) or variations thereof, e.g. birds use a female heterogametic system (males are ZZ and females are WZ). However, other vertebrate species have acquired an array of sex determination systems that do not rely exclusively on these classical chromosomal systems. In this respect, fish are one of the most intriguing groups that are presently under study since, aside the classical sex chromosomal XX/XY or WZ/ZZ systems, they can also display a diverse array of sex determining mechanisms that include different forms of hermaphroditism and environmental sex determination (see Devlin and Nagahama, 2002 for review). Thus, to date, no simple genetic sex determining system can be generalized for fish.

In mammalian, the presence of a Y chromosome is sufficient to determine the genetic fate of the gonad. Indeed, the presence of a Y-chromosome specific gene, named *SRY*, is sufficient to activate male gonadal development. Conversely, the absence of this gene precludes male sexual development and thus, female gonads develop, which has long been seen as a default mechanism. To date, all efforts for identifying an *SRY*-homolog or functionally equivalent gene in fish have failed. However, recently, a male sex determining gene, named *dmY/dmrtY*, has been identified in Medaka, *Oryzias latipes*, which is functionally equivalent to the mammalian *SRY* (Kobayashi et al., 2004). However, so far, this gene is absent in all other fish genomes, suggesting that Medaka has acquired this male sex determining gene in a recent event and that is species specific (Vollf et al., 2003). Nonetheless, although no general sex determining gene has been found in fish, in the last few years different sex-specific probes have been obtained, each one of them shown to be specific to each species and even sometimes to a particular population (Forbes et al., 1994; Nakayama et al., 1994; Reed et al., 1995).

Different sex determining models have been proposed in fish, namely models based on multiple sex chromosomes, polygenic sex determination and autosomal influence (Reinboth, 1983; Chourout, 1994). However, there is still scarce information about the molecular mechanisms involved in these processes. Moreover, an additional level of complexity arises in species that possess a sex determining system that is further modulated by environmental factors. Indeed, sex determination in different fish species (*Oreochromis niloticus*, *Oreochromis aureus*, *Odonthestes bonariensis*, *Dicentrarchus labrax*), has been found to be sensitive to temperature (Baroiller et al., 1995; Strüssmann,

1997; Desprez and Melard, 1998; Pavlidis et al., 2000). Interestingly, the influence of temperature appears to differ amongst species and appears not to be sufficient to induce complete female or male monosex populations. Indeed, there is evidence to suggest that the genetic background is not completely overridden by environmental cues, and that there are individual differences in susceptibility to temperature. Furthermore, different reports for example in the European sea bass, *Dicentrarchus labrax*, have shown combined effects of temperature and parental influence (Saillant et al., 2002) or genetic strain (Mylonas et al., 2003; Mylonas et al., 2005), but have also failed to produce 100% female or male populations.

Despite the sex determining system used by the different fish species, and lack of a sex determining gene, it is still hypothesized that most factors involved within the sex determination cascade are probably conserved among species. In fact, in the past years, different factors involved in mammalian sex determination, e.g., *DMRT-1*, *SOX9*, and *WT-1*, have also been found in fish, suggesting a conserved role throughout evolution (Kent et al., 1995; Kondo et al., 2002; Nakamoto et al., 2005). Nonetheless, the exact functions of these genes are still unclear in fish.

One of the genes that is a strong candidate for a sex determining role in fish is *Ahch* or *DAX1* (Dosage-sensitive sex-reversal, AHC, on the X chromosome, gene 1). This gene was originally isolated in the X-chromosome of patients presenting a complex syndrome called adrenal hypoplasia congenital (AHC) (Zanaria et al., 1994). Interestingly, this gene was found to be located within an X-linked locus that overlapped the AHC locus which, when duplicated, results in male sex reversal (Bardoni et al., 1994; Zanaria et al., 1994). After the identification of this gene, different reports have established a direct link between *DAX1* mutations and clinical signs of AHC (Yu et al., 1998b). A role of *DAX1* in mammalian sex determination was further emphasized when it was established that the gene is repressed by *SRY* during male sex determination, linking it to ovary formation (Swain et al., 1998). As such, it has been regarded as an anti-testis gene in mammalian. Interestingly, *DAX1* homolog genes have been identified in other vertebrate species, namely in bird and reptile embryos and fish larvae during the sex determining period, suggesting that it may also be involved in sex determination of lower vertebrates (Smith et al., 2000; Torres-Maldonado et al., 2002; Wang et al., 2002).

A key feature of fish sex determination that differentiates it from mammalian sex determination is that steroids are required for both female and male sex differentiation (Baroiller et al., 1999; Devlin and Nagahama, 2002). Indeed, exogenous steroid administration, steroidogenic enzyme inhibitors or steroid receptor antagonists can modify sex during sex differentiation or even in some cases during adulthood (Devlin and Nagahama, 2002). Several of the factors involved in the sex differentiation cascade,

including DAX1, can also target steroidogenic enzymes and/or steroid receptors suggesting possible mechanisms of action (see below). Thus investigations into the structure, regulation and mechanism of action of candidate genes like *DAX1* can shed some light on the sex differentiation cascade in fish and how it can be modified by exogenous.

1.2 *DAX1* gene and protein structure

The human *DAX1* gene has a very simple genomic structure composed of two exons separated by a single intron (Zanaria et al., 1994). This gene encodes a 470 amino acid protein (Zanaria et al., 1994). Recently, however, a 401 amino acid DAX1 protein, named DAX1 α , was identified in humans, as a result of transcription of exon 1 and of the transcription of a cryptic exon that was previously not identified within the intronic sequence of the *DAX1* gene (Hossain et al., 2004).

The DAX1 protein has high similarity to other members of the nuclear receptor superfamily. However, due to the lack of known ligands, it has been classified as an orphan member of the nuclear receptor superfamily, and named *Nr0B1* (Burris et al., 1995). Nuclear receptors are composed of four modules: the A/B domain, the C domain or DNA-binding domain (DBD), D domain or the hinge region, and E domain or ligand-binding domain (LBD) (Guiguère, 1999). The *DAX1* gene has a carboxy-terminal domain composed of a homologous LBD to other nuclear receptors and an AF-2 transactivation domain similar to those found in other nuclear receptor members. However, it lacks the conventional DBD, domain A/B and hinge region. Indeed, the DAX1 amino-terminal domain has an atypical structure composed of 3.5 repeats of a 65–70 amino acids long cysteine-rich motif that has no known homology to any other proteins, with the exception of the small heterodimer partner (SHP), encoded by *NR0B2*, which is the only known DAX1 family member. Another striking feature of this novel DBD is that it also lacks the classical zinc fingers found in other nuclear receptors and, instead, it possesses 3 LXXLL-like motifs within each of the 65-70 amino acid repeat, that are used for interaction with other receptors (Zhang et al., 2000; AgoulNIK et al., 2003; Kawajiri et al., 2003). The C-terminal domain of DAX1 has strongest amino acid similarity to the LBD of the COUP-TF and retinoid X receptor (RXR) (Zhang et al., 2000). Interestingly, this domain has been shown to be responsible for transcriptional silencing activity in mammalian species and is only found in a subset of other members of the nuclear receptor super family: thyroid hormone (TR), the related oncogene product v-erbA, retinoic acid receptor (RAR), and the chicken ovalbumin upstream promoter transcription factor (COUP-TF) (Lalli et al., 1997). The silencing activity of DAX1 is thought to be due to a bipartite domain in the C-terminal

(Lalli et al., 1997) that corresponds to the helix 3 and helices 11-12, according to the DAX1 protein structure prediction when aligning DAX1 with RAR, TR and v-erbA sequences (Lalli et al., 1997; Altincicek et al., 2000). Nonetheless, overall, the DAX1 structure is more similar to SHP, since the C-terminal region (LBD) of both proteins are well conserved both lack the typical nuclear receptor DBD that is replaced by one of the 3.5 65–70 amino acid repeat in the N-terminal domain (DBD) (Seol et al., 1998).

1.3 DAX1 molecular function

In patients with AHC, all *DAX1* mutations localize to the C-terminal LBD, which significantly reduce its repressive effects in cell culture assays (Achermann et al., 2001a), and thus implicating the LBD as a mediator of repression. Indeed, a bipartite transcriptional silencing domain has been shown to exist in the LBD, which interacts directly with co-repressors to mediate repression. Additionally, a direct role for the N-terminal LXXLL motifs and for the LBD AF-2 core have also been shown in mediating repression of different nuclear receptors, namely through direct interaction with target genes: estrogen receptors (Er α and Er β), androgen receptor (AR), progesterone receptor (PR), *NR4A1* encoding Nur77, and *NR5A2* encoding liver receptor homologue-1 (LRH1) (Zhang et al., 2000; Holter et al., 2002; AgoulNIK et al., 2003; Suzuki et al., 2003; Song et al., 2004). DAX1 transcriptional silencing activity can involve direct protein–protein interactions between DAX1 with known co-repressors, namely Alien, NcoR and MLK2, and RIP140 (Crawford et al., 1998; Altincicek et al., 2000; Sugawara et al., 2001; Eckey et al., 2003). Recruitment of co-repressors has been shown to be a key factor in the regulation of Steroidogenic factor 1 (SF1), a key regulator of steroid hydroxylase enzymes (Lala et al., 1995; Morohashi and Omura, 1996). DAX1 protein interacts with DNA-bound SF1 via the DAX1 N-terminal domain, with the subsequent recruitment of co-repressors to the promoters of target genes, via the DAX1 C-terminal transcriptional silencing domain (Crawford et al., 1998). In cultured cells, DAX1 represses SF1-mediated transcriptional activation of steroidogenic genes, such as *Cyp11A*, *Cyp17* and *Cyp19* (Lalli and Sassone-Corsi, 2003). Additionally, when over expressed, DAX1 markedly impairs steroidogenic output and transcriptionally represses the gene promoters of other steroid enzymes such as StAR and 3 β -HSD (Lalli et al., 1997; Zazopoulos et al., 1997; Jo and Stocco, 2004). Moreover, DAX1 is also able to repress SF1-mediated up regulation of the gene encoding anti-Müllerian hormone (AMH, also known as Müllerian inhibiting substance; MIS) during male sexual differentiation, either through repressed synergy between GATA4–SF1 or between SF1–WT1, during activation of the *AMH* promoter (Nachtigal et al., 1998; Tremblay and Viger, 2001). DAX1 is also able to repress the synergy between SF1–EGR1

and block the GnRH stimulation of the *LHβ* promoter (Dorn et al., 1999). Thus, collectively, these data suggest that DAX1 is a global negative regulator of genes involved in steroid hormone production and metabolism in endocrine tissues.

Although most of the repressive actions of DAX1 that have been described involve protein: protein interaction with co-repressors, the initial report on DAX1 isolation and characterization has demonstrated that DAX1 could bind directly to the retinoic acid-responsive element (RARE) *in vitro* (Zanaria et al., 1994). However, other studies support the view that DAX1 binds to particular nucleic acid structures, rather than to particular sequences. Indeed, DAX1 has been shown to bind to DNA loops in target gene promoters. The StAR and *DAX1* own promoters are two of the examples that have been shown to contain DNA hairpin sequences that are bound by DAX1 (Zazopoulos et al., 1997). The DAX1 protein has not only been shown to regulate its own promoter, as it has also been shown to homodimerize with other DAX1 molecules (Iyer et al., 2006). The motifs involved in DAX1 homodimerization include the LXXLL domains and AF-2 domain. Altogether these data suggest an auto-regulatory feedback loop on its own transcription.

DAX1 is a nucleo-cytoplasmatic shuttling protein that has been shown to be associated with ribonucleoprotein structures and polyribosomes in the cytoplasm, suggesting that DAX1 might also act at a post-transcriptional level, through direct binding to RNA and polyribosomes and thus may be acting as a shuttling protein for export of RNA species into the nucleus (Lalli et al., 2000). Nuclear import requires the integrity of a bipartite transcriptional repression domain in the DAX1 C-terminus. However, in AHC mutations, DAX1 nuclear localization is impaired as all DAX1 mutant proteins are retained within the cytoplasm (Lehmann et al., 2002).

1.4 *DAX1* gene expression and regulation

DAX1 gene expression has been detected in the developing adrenal cortex, gonad, anterior pituitary and hypothalamus, in adult adrenal cortex, Sertoli and Leydig cells in the testis, and in theca, granulosa, and interstitial cells in the ovary (Guo et al., 1996; Ikeda et al., 1996; Swain et al., 1998). Moreover, *DAX1* mRNA expression is coincident with that of SF1 in most tissues and developmental stages in mammalian, further suggesting that these two transcription factors are network partners (Ikeda et al., 1996; Ikeda et al., 2001). In addition, different studies have shown that the transcriptional regulation of SF1 target genes during development and in the adult organism appear to be regulated by the intracellular levels of SF1 and DAX1, with the ratio of these two factors determining whether the target genes are activated or repressed. Thus, if higher levels of SF1 than

DAX1 exist, the target genes will be activated, whereas, if more DAX1 is present, the target genes are either not activated or repressed.

In addition to the importance of SF1-DAX1 ratio, both human and rodent *DAX1* promoters contain extended AGGTCA-like half sites that are recognized and bound by SF1 (Burris et al., 1995; Yu et al., 1998a). Indeed, SF1 has been shown to use these binding sites to regulate *DAX1* transcription in endocrine tissues. Interestingly, the ERR family (estrogen-related receptors, type α , β , γ), which is composed of 3 orphan nuclear receptors, have been shown to bind to the same consensus binding site as SF1. In fact, one of these members, ERR γ , has been demonstrated to bind to the SF1 response element in *DAX1* promoter (Park et al., 2005).

Another set of candidate regulators of *DAX1* transcription are the GATA transcription factor family genes. Putative GATA binding sites have been found in the mammalian *DAX1* promoter (Hoyle et al., 2002), although to date no direct interaction of DAX1 has been reported with GATA elements alone. However, GATA-4 and GATA-6 have been shown to play a role in male sex determination, steroidogenesis, and possibly cell survival (see la Voie, 2003 for review) and to synergize with SF1 in steroidogenic tissue (Nachtigal et al., 1998), thus acting within the same processes as DAX1.

One of the key regulators of *DAX1* transcription is Wilms tumor 1 (*WT1*). *WT1* expression precedes that of *DAX1* in the urogenital ridge, and at the protein level, both DAX1 and WT1 show an overlapping spatial expression profile in the developing fetal gonad (Kim et al., 1999). Moreover, WT-1 can individually activate *DAX1* transcription through direct binding to its promoter or through recruitment of the co-activator FHL2 (Kim et al., 1999; Du et al., 2002). In addition, WT-1 is also able to activate *SF1* transcription individually or through recruitment of co-activator Lhx9 (Wilhelm and Englert, 2002). Thus, WT-1 has been proposed as an upstream regulator of SF1 and DAX1 in mammalian sex determination cascade.

Activation of the WNT-beta catenin signaling pathway has been shown to alter *DAX1* transcription. WNT ligands bind to cell-surface Frizzled receptors, initiating a cytoplasmatic signaling cascade that results in beta-catenin stabilization, cytosolic accumulation, and subsequent translocation to the nucleus. Within the nucleus, beta-catenin classically complexes with a member of the T-cell Factor (TCF) family of transcription factors to activate target-gene transcription (Mulholland et al., 2005). Upon Wnt4 stimulation, beta-catenin activates *DAX1* transcription either through direct interaction with the TCF/LEF binding sites on its promoter or through direct interaction with SF1 on the *DAX1* promoter, in a TCF/LEF independent process (Mizusaki et al., 2003).

1.5 The role of DAX1 in sex determination

1.5.1 Early embryonic development

Three germ cell layers, the mesoderm, endoderm and ectoderm, are formed during embryonic development. The endoderm develops into the digestive tract; the ectoderm, into skin and the central nervous system and the mesoderm develops into the internal organs. Gonads are formed by cells originating in different locations: Somatic cells originate from proliferation of cells within the genital ridge itself, and primordial germ cells (PGCs) originate in the proximal epiblast. Upon the activation of two genes, *c-kit* and *steel*, PGCs start to migrate from the posterior end of the primitive streak into the allantois and then into the adjacent embryonic endoderm, which first forms the gut (Anderson et al., 2000). Here, a cluster of PGC will recover their active migratory capacity and move towards the genital ridges. During migration and early settlement in the genital ridge, PGCs express several markers, e.g., tissue- SF1 (*AP*) (MacGregor et al., 1995), and the transcription factor *Oct3/4* (Yeom et al., 1996). Once in the genital ridge, PGCs start to express different gene markers, within which, the vasa protein will ultimately activate somatic cell development in the genital ridge (Toyooka et al., 2000).

Once PGCs begin to proliferate and establish in the ventral region of the urogenital ridge, they undergo active proliferation (Tam and Snow, 1981; Godin et al., 1990). At this stage, PGCs lose their motile behavior (Wylie et al., 1969) and together with proliferating coelomic and mesenchymal cells, form a cluster of condensed cells that gradually becomes the undifferentiated gonad and differentiate into germ stem cells that divide by mitosis to produce and originate the gametes. In the female, once mitotic proliferation stops, retinoic acid activates the *stimulated by retinoic acid 8 (stra8)* gene in germ cells, and these initiate meiotic differentiation (Koubova et al., 2006). In males, however, expression of a retinoid-degrading enzyme known as CYP26B1 blocks the retinoic acid stimulation and results in germ cells arrest in the G0/G1 stage of the mitotic cell cycle (McLaren et al., 2004) until after birth. Thus, in the absence of CYP26B1, male germ cells enter meiosis prematurely as in a normal ovary (Bowles et al., 2006).

Embryonic germ (EG) cells, derived from cultured primordial germ cells (PGCs), are the embryonic precursors of the gametes of the adult animal. The pluripotency of EG cells has been demonstrated in widely-used assays and demonstrate that they share many properties with pluripotent embryonic stem (ES) cells (Donovan and Gearhart, 2001). *DAX1* was found to be expressed in early pre-implantation embryos as well as in ES cells (Clipsham et al., 2004), together with other genes involved in sex determination, including *SF1*, and *WT-1*. In pre-implantation embryos, *DAX1*, *SF1*, and *WT1* are

expressed in the inner cell mass (Clipsham et al., 2004). In fact, upon implantation, *DAX1* is specifically expressed and can function as a marker for lineage determination, separating the extra embryonic visceral endoderm (VE) from the embryonic layers (Niakan et al., 2006). Interestingly, differentiation of ES cells with stimuli in the ES cell media has also been reported to result in loss of *DAX1* RNA similar to that observed for *Oct-4*. Disruption of the expression of *DAX1* by siRNA knockdown as well as a conditional knockout in ES cells caused their differentiation (Niakan et al., 2006), suggesting that *DAX1* is necessary for the maintenance of pluripotent state of stem cells. Additional proof for this *DAX1* novel role was obtained from the demonstrated interaction with Nanog, a marker of pluripotency, in ES (Wang et al., 2006). Altogether, these results show that *DAX1* is determinant for the early mammalian embryonic development and development of embryonic germ cell lineages.

1.5.2 Early gonad development

Genes that are important in the differentiation of the intermediate mesoderm and the urogenital system as a whole will generally have a role in early gonad development. This group of genes is characterized by their mutant phenotypes, which show defects in both early kidney, adrenal and gonad development. A growing list of genes involved in this process has been identified, namely *WT-1*, *SF1*, *Lim1*, *Lim-9* and *EMX2*. *WT-1* was found to be determinant for the development of genital ridge, adrenals and kidney in mouse embryos (Pritchard-Jones et al., 1990; Kreidberg et al., 1993). Likewise, *SF1* is also expressed in the developing urogenital ridge and undifferentiated gonad while the *SF1* knockout in mouse embryos prevents the establishment of undifferentiated gonads and all *SF1* null mutants develop a female phenotype (Luo et al., 1994). *Lim-1*, *Lim-9* and *Emx2* are also required for development of the urogenital system (Shawlot and Behringer, 1995; Miyamoto et al., 1997; Birk et al., 2000). Once the indifferent gonad is formed, arrays of autosomal genes are activated in order to establish the genotypic sex of the bipotential gonad either into a female or into a male. In mammalian, the presence of the Y chromosome gene, sex-determining region of the Y-chromosome gene or *SRY*, is sufficient to trigger differentiation of testes from the indifferent gonad (or genital ridges) that would otherwise develop as ovaries (Gubbay et al., 1990; Sinclair et al., 1990). Upon *SRY* activation, pre-sertoli cells start to develop and *SOX9* is expressed (Kent et al., 1995). Yet, although it is not known whether *SOX9* responds directly or indirectly to *SRY*, gain-of-function studies in mice and humans (reviewed in Canning and Lovell-Badge, 2002) indicate that *SOX9* may be sufficient for male sex determination even in the absence of *SRY*.

In mammalian, *DAX1* is expressed during early ovarian development but is suspended during testicular formation (Swain et al., 1996), and has also been shown to be repressed by SRY during testicular development, which implies a critical role for this gene in ovarian formation (Swain et al., 1998). (Swain et al.) (1998) using a transgenic mouse model (*Mus musculus* origin) carrying extra copies of *DAX1* have tested whether *DAX1* function in sex determination was dosage dependent. Surprisingly, when these mice were bred, they did not observe sex reversal in the offspring, unlike what is seen in human patients with *DAX1* duplication in their genome (Bardoni et al., 1994). However, mice with the highest SF1 expression (up to five times that of normal levels) exhibited retarded testis formation, suggesting that *DAX1* functioned as a female promoting gene or as an anti-testis gene. The function of *DAX1* in ovarian development is still unclear. Although *DAX1* expression is detected in the developing ovary at various stages of development homozygous *DAX1*-deficient female mice are normal and fertile, except for slight ovarian follicular defects, suggesting that *DAX1* is not required for ovarian development (Yu et al., 1998b). In addition, female patients with AHC do not present gonadal defects, although later in puberty some patients present compromised endocrine function (Seminara et al., 1999). Thus, these studies suggest that *DAX1* is more important for female gonadal endocrine function than for its morphological development.

In contrast, studies with *DAX1*-deficient male mice provided irrefutable evidence that *DAX1* is necessary for proper testicular development and function, in opposition to an anti-testis function. Indeed, the *DAX1*-deficient mice are hypogonadal and infertile, and have testicular and spermatogenic defects with loss of germ cells and degeneration of the seminiferous epithelium (Yu et al., 1998b). In addition, the *DAX1*-deficient XY mice develop as phenotypic females when in the presence of a weakened *SRY* allele, further emphasizing a crucial role in testis determination (Meeks et al., 2003b). Further characterization of the *DAX1*-deficient mouse model has also shown that this gene is determinant for testis cord organization during development (Jefferis et al., 2001a; Meeks et al., 2003a), and that the lack of *DAX1* expression in Sertoli and Leydig cell leads to infertility and low sperm counts, a condition that is only partially rescued when *DAX1* expression is allowed in these cells (Jefferis et al., 2001b). However, expression of *DAX1* in each of these cell types individually is not sufficient to rescue the testicular pathology, suggesting that *DAX1* function in both Sertoli and Leydig cells (in addition to other somatic cell types) is necessary for proper testicular development (Jefferis et al., 2001b; Meeks et al., 2003b). Interestingly, a clear evidence that *DAX1* is not an anti-testis gene, but acts instead as 'pro-testis' gene was obtained recently, when *DAX1*-knockout mice were XY sex reversed after they were crossed with the *M. d. poschiavianus* strain containing the Y chromosome *Sry*^{POS} allele (Meeks et al., 2003b). These *Dax1*^{-/-}Y^{POS} mice lacked

testicular cords, Sertoli and Leydig cell markers and were sex reversed. Interestingly, *SOX9* expression was significantly reduced in these mice even in the presence of normal *SRY* levels. These results imply that *DAX1* is determinant for male sex and also that it is acting downstream the *SRY* but in concert with *SOX9*.

Altogether, these studies provide clear evidence for a determinant role of *DAX1* in early embryonic development and in male gonadal development. Reduction or increase in *DAX1* expression has no apparent major effect in female development but, in humans and rodents, *DAX1* dosage can influence the final outcome of the phenotypic gonads in genetic males.

1.6 The role of *DAX1* in sex differentiation

Once pre-sertoli cells start to develop, anti-müllerian hormone (AMH), a secreted peptide, starts to be produced (Teixeira et al., 2001) upon the activation of *SRY*, *SOX9*, *WT1*, *SF1* and *GATA4* (Viger et al., 2005). This peptide causes regression of the Müllerian ducts, which otherwise would give rise to the female morphological structures. Thus, Sertoli cells organize into the testicular cords, where spermatogenesis later takes place, and are involved in the support of spermatid differentiation. Another cell lineage, the Leydig cells also starts to differentiate with the primary function of initiating steroidogenic capacity and to synthesize testosterone. Testosterone production will support the maintenance of the Wolffian ducts, which develop into the different structural components of the male reproductive tract. Thus, while testicular development is initially independent of steroids, the later proliferation of Sertoli cells and full masculinization requires testosterone (Sharpe, 2006).

The development of the female reproductive tract has received much less attention than the male tract, probably because of the widespread notion that it occurs 'by default' in the absence of *SRY*. Although to date no sex determining gene has been found in females, repressor proteins preventing male differentiation are expressed in the ovary, e.g. *WNT-4* (a member of the Wnt/ Wingless family). In early development, *WNT-4* suppresses Leydig cell differentiation and the synthesis of testosterone, probably by repressing *SF1* function or inhibiting migration of steroidogenic precursor cells into the developing ovary (Fleming and Vilain, 2005). Female *WNT-4* null mice develop virilized gonads and Wolffian derivatives (see Park and Jameson, 2005 for review), demonstrating an essential role in female sex differentiation. Moreover, other genes are also essential for the development of granulosa cells and oocytes within the ovary, namely *FOXL2* and *FIGX* (Löffler and Koopman, 2002; Fleming and Vilain, 2005). Interestingly, *DAX1* is involved in the inhibition of *AMH* expression, through directly inhibiting the binding of key regulators

to its promoter, e.g. *SF1* and *GATA-4* (Nachtigal et al., 1998; Tremblay and Viger, 2001). Moreover, *DAX1* has been shown to be up regulated by *WNT-4* through interaction with *SF1* (Jordan et al., 2001). In addition, both *DAX1* and *WNT-4* are up regulated by *WT-1* in female gonads (Sim et al., 2002), suggesting that in concert, *DAX1* and *WNT-4* are part of a cascade of genes controlling the development of the Müllerian tract and additional morphological structures in the female reproductive system (Rey et al., 2003). Overall, although the activation of AMH secretion is determinant for male sexual differentiation, by itself is not sufficient, since males need to acquire steroidogenic capacity in order to pursue masculinization of the gonads. On the contrary, the inhibition of AMH secretion and steroidogenic function in the early gonad enables the Wolffian Tract regression and the Müllerian tract development into the morphological structures of the female reproductive system. Thus, it is clear that steroid production plays a determinant role in sexual differentiation in mammalian, but also in fish (see below and e.g. Devlin and Nagahama, 2002).

1.6.1 Regulation of steroidogenic development of the gonads

The enzymatic steps and their genetic control in the testicular biosynthesis of testosterone from cholesterol are well documented (reviewed in Payne and Hayes, 2004). The synthesis of steroids from cholesterol involves trafficking between mitochondria and smooth endoplasmic reticulum, mainly via P450 or CYP enzymes. All steps are necessary for androgen production but key points include the rate limiting step controlled by the steroidogenic acute regulatory protein (StAR). In mammalian, the main enzymes involved in the production of testosterone are cytochromes P450 cholesterol side-chain cleavage (P450_{scc} or CYP11A), the 3 β -hydroxysteroid dehydrogenases (3 β -HSD1-6) types 1–6, and 17 β -hydroxylase 17–20 lyase (CYP17). The enzymes 17-hydroxysteroid dehydrogenases, types 1–3 (17 β -HSD 1-3) and 5 α -reductase function to amplify the androgenic signal through the synthesis of the more potent androgens, testosterone and dihydroxytestosterone (DHT). The expression pattern of *CYP* genes correlates with that of the *SF1* (Honda et al., 1993). *SF1* is an orphan receptor that binds to extended AGGTCA-like half sites present in all steroidogenic *CYP* proximal promoters and stimulates their expression (Lala et al., 1995; Morohashi and Omura, 1996). Interestingly, *SF1* and *DAX1* are co-expressed in steroidogenic tissues as well as in the hypothalamus and pituitary, early in development (Ikeda et al., 1996). However, when overexpressed, *DAX1* markedly impairs steroidogenic output and transcriptionally represses the promoters of *SF1* target genes such as *StAR*, *Cyp11A*, *3 β -HSD*, *Cyp17* and *Cyp19* (Lalli et al., 1997; Zazopoulos et al., 1997; Sim et al., 2002; Lalli and Sassone-Corsi, 2003; Jo and Stocco, 2004). Thus,

SF1 and DAX1 are suggested to be antagonist regulators of steroid hormone production, and further emphasize the repressive actions on steroid production in endocrine cells.

The initial production of testosterone in Leydig cells is independent of LH secretion, because LH only becomes detectable in the pituitary latter (El-Gehani et al., 2000). In fact, genetic mutant or knock-out male mice deficient in gonadotropin releasing hormone (GnRH), luteinizing hormone (LH) or LH receptors (LHR), all exhibit, although with varying degrees, prenatal masculinization (El-Gehani et al., 2000; Zhang et al., 2001) which corroborates that activation of testosterone production is gonadotropin independent. Upon activation of gonadotropin production in the pituitary, testosterone synthesis in the developing testes is under strict control of chorionic gonadotropin (hCG) and LH (Misrahi et al., 1998). Both hCG and LH stimulate testosterone synthesis via the LH receptor (LHR) in the testis. Mutations in the *LHR* are associated with gonadotropin unresponsiveness and lead to Leydig cell agenesis and, subsequently, to defective sexual differentiation. More often, inactivation of *LHR* result in a completely female phenotype, although incomplete virilization due to partial receptor responsiveness with subnormal androgen synthesis has also been described (Misrahi et al., 1998). These molecular abnormalities imply an active role for the LHR in Leydig cell growth and differentiation. *DAX1* is expressed in the developing pituitary structure by the time it starts to be expressed in the urogenital ridge (Swain et al., 1998; Ikeda et al., 2001). In addition, *DAX1* is expressed throughout development in different structures of the hypothalamus-pituitary-gonadal-adrenal axis (HPGA) (Swain et al., 1998; Ikeda et al., 2001), suggesting an active role in development and early function of this axis. Moreover, patients with *DAX1* mutations, presenting hypothalamic hypogonadism (HHG) with absent or delayed puberty, also present impaired or absent production of GnRH from the hypothalamus and/or of FSH and LH from the anterior pituitary (Vaidya et al., 2000; Achermann et al., 2001b) compromising latter steroid production when they reach puberty.

Interestingly, gonadotropins have been shown to regulate *DAX1* expression in the gonads (Tamai et al., 1996; Yazawa et al., 2003). In early development of Sertoli cells, *DAX1* expression peaks during the first synchronized spermatogenic wave, and returns to basal levels thereafter (Tamai et al., 1996). Sertoli cells contain specific G-protein-coupled receptors for the pituitary hormone FSH that upon binding stimulates adenylate cyclase activity and leads to increased concentrations of intracellular cAMP (Heckert and Griswold, 2002). Interestingly, *DAX1* expression is significantly down-regulated upon FSH treatment and by treatment with the cAMP analog, (Bu) cAMP, which bypasses the FSH receptor and leads directly to activation of the protein kinase A pathway (Tamai et al., 1996). SF1 is a transcriptional regulator of cAMP-induced genes (e.g. steroid hydroxylases) and is also phosphorylated by cAMP-dependent protein kinase (PKA) (Zhang and Mellon, 1997).

Moreover, PKA stimulation potentiates SF1 function while prevents DAX1 antagonistic interaction with SF1. Moreover, increased cAMP levels have been shown to up-regulate SF1 levels and down-regulate DAX1 levels (Osman et al., 2002). Thus, activation of PKA signaling pathway by gonadotropins stimulate SF1 levels, down regulates DAX1 levels, induces DAX1 and SF1 dissociation, and thus, promotes SF1 interactions with coactivators and activation of SF1 target genes (e.g. P450 steroid hydroxylases) (Tamai et al., 1996; Osman et al., 2002). One of these target genes is the enzyme that is responsible for the aromatization of androgens into estrogens, the cytochrome P450 aromatase (P450arom), the product of the *CYP19* gene. The *CYP19* promoter activity in male gonads is up-regulated by FSH/LH and through activation of the cyclic AMP-dependent signaling pathway promotes the activation and individual binding of *SF1* to promoter response elements, and/or recruitment of beta-catenin (Genissel et al., 2001; Parakh et al., 2006). Interestingly, the expression of the *CYP19* gene, is increased significantly in Leydig cells isolated from DAX1 mutant mice (Wang et al., 2001), suggesting a repressive action of DAX1 on the *CYP19* promoter. Furthermore, DAX1 was shown to block the cAMP-SF1 pathway-dependent P450arom expression (Gurates et al., 2002). Altogether, these studies show that gonadotropins activate the cAMP-PKA signaling that will lead to SF1 phosphorylation. Subsequently, *SF1* increased levels and transcriptional activity suppress *DAX1* transcription and activate steroid enzymes transcription and activity, leading to increased steroid production. Thus, it is clear that the balance of *SF1* and *DAX1* levels within steroidogenic tissues is determinant for steroid production, and suggests a determinant role for both transcription factors in the activation and regulation of steroid production in gonads during sexual differentiation.

Interestingly, DAX1 is not only able to modulate steroid production but is also able to regulate androgen and estrogen action, through physical interaction with both androgen receptor (AR) and estrogen receptors (ERs), resulting in repressed activity (Ikonen et al., 1997; Zhang et al., 2000; Holter et al., 2002). Altogether, it is clear that DAX1 is determinant for gonadal sexual differentiation as it regulates androgen and estrogen production and is also able to regulate their action in the cell through direct protein interaction with both AR and ERs. A general model summarizing DAX-1 interactions is shown in figure 1.

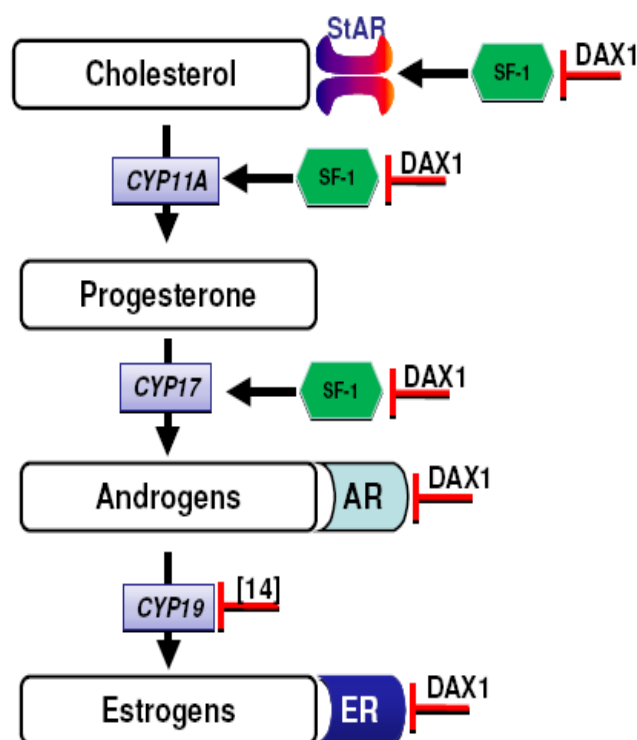


Figure 1– Schematic representation of pathway for the biosynthesis of sexual steroids. DAX1 regulates key steps for androgen production by repressing the transcriptional activation of SF-1 on the promoters of *StAR* (Lalli et al., 1997), *CYP11A* (Lalli et al., 1998) and *CYP17* (Hanley et al., 2001). DAX1 binds directly to the *CYP19* promoter to downregulate its transcription (Wang et al., 2001). It also regulates estrogen (Zhang et al., 2000) and androgen receptors (Holter et al., 2002) through protein-protein interactions. *CYP11A*, *CYP17* and *CYP19* are, respectively, genes that encode for the P450 enzymes cholesterol side chain cleavage, 17- hydroxylase and aromatase. *StAR* is the mitochondrial cholesterol transporter protein. AR and ER are respectively androgen and estrogen receptors (α and β).

1.7 The role of DAX1 in fish

In fish, the *DAX1* homologue gene has been recently characterized in zebrafish (*Danio rerio*) and tilapia (*Oreochromis niloticus*) (Wang et al., 2002; Zhao et al., 2006). The *DAX1* gene structure in both fish species is conserved to that described in mammalian, as they are also composed of 2 exons and 1 intron. The first exon codes for the putative DBD and most of the LBD of the protein, while the second exon, which is a lot smaller, codes for the rest of the LBD and AF-2 core. Thus, the gene structure and main structural features (DBD, LBD and AF-2 core) are also present in fish DAX1 proteins.

Interestingly, the putative DBD of DAX1 fish proteins is also atypical since it does not possess the classical zinc fingers of other nuclear receptor members, but instead, they contain LXXLL-like motifs as identified in mammalian homolog genes. However, unlike mammalian DAX1 proteins, the putative DBD is considerably smaller since it lacks the 3.5 repeat regions. Instead, the DAX1 proteins contains only one of the 3 LXXLL-like motifs found in mammalian DAX1 proteins, resembling the DBD structure of its family member, small heteroprotein (SHP). However, to date, there is no information on whether this motif is sufficient for enabling protein interactions with other receptors as described in mammalian.

Fish *DAX1* genes are expressed within the HPAG axis as described in mammalian (Wang et al., 2002; Zhao et al., 2006). However, unlike mammalian, *DAX1* expression is also detected in most of the tissues of adult fish tested so far, contrasting the typical HPAG axis distribution reported earlier in human and rodents (Wang et al., 2002). In addition, tilapia *DAX1* is expressed at similar levels in both adult female and male gonads, which contrasts the dimorphic pattern described in mammalian (Wang et al., 2002). Although there is still no data demonstrating a role for DAX1 in fish sex determination, the zebrafish, tilapia and trout (*Oncorhynchus mykiss*) *DAX1* genes are expressed during the sex determining period (Wang et al., 2002; Baron et al., 2005; Zhao et al., 2006). Indeed, a recent report has suggested a role for DAX1 in trout testicular differentiation (Baron et al., 2005). Interestingly, Wang et al. (2002) have identified the fugu (*Takifugu rubripes*) *DAX1* homolog gene within the genome database, and surprisingly, have also identified a putative duplicate of the *DAX1* gene (which was named *DAX2* or *Nr0B1b*) that is also found in tilapia (Accession number ABB88832). Nonetheless, to date there is still no information on this duplicate gene in either fugu or tilapia.

In zebrafish embryos, *DAX1* mRNA has been detected in head kidney, central nervous system, liver, brain and in trunk midline region between 4 and 32 days post fertilization (dpf), suggesting that DAX1 is involved in early embryonic development, well before gonads start to be developed (Zhao et al., 2006). Surprisingly, target disruption of *DAX1* in zebrafish resulted in impaired osmoregulation, suggesting that it has a determinant role in early adrenal function and glucocorticoid production in fish larvae (Zhao et al., 2006).

1.8 Aim of the thesis

The *DAX1* gene has been identified in two different fish species. Despite the high conservation in sequence and gene structure, it is still unknown whether this gene is a sex determining gene as described in mammalian. Thus, the aim of this thesis was to study

the *DAX1* and *DAX2* expression during the sex determining period and also throughout the gonadal sex differentiation period. In addition, to shed some light on the mechanisms underlying the regulation of *DAX1* expression in fish, we have aimed to identify the *DAX1* promoter in fish and, based on promoter conservation between fish and mammalian, to identify putative regulatory sequences within its promoter that could help to identify possible gene network partners. Finally, we have sought to explore a possible role of *DAX1* in male gonadal steroidogenesis as a validation of one of the putative gene networks that have resulted from the promoter analysis.

For this purpose, the organization of this thesis will be as follows:

In **Chapter 2**, the European sea bass *DAX1* (*sbDAX1*) gene and coding sequence are described. Protein structure and evolutionary conservation of *DAX1* protein was also assessed. The mRNA expression in adult fish was analyzed in both female and male tissues. Within this chapter, the *DAX1* mRNA expression was also studied during the temperature sex determining period (TSD) and during the gonadal sex differentiation period (GSD).

In **Chapter 3**, we describe the isolation of a duplicate *DAX1* gene, named *DAX2*, and of another *DAX1* family member named *SHP*. For both genes, we have studied the gene structure, sequence and evolutionary conservation. In addition, we have also studied the mRNA expression of these novel members during the GSD and TSD periods described for *DAX1* in chapter 1. In addition, we have also analyzed the expression of *SF1* and of two *SOX9* duplicate genes (*SOX9.1* and *SOX9.2*) in order to clarify which genes are involved in the temperature sensitive sex determining cascade in sea bass TSD.

In **Chapter 4**, we describe the isolation of the sea bass *DAX1* putative promoter sequence. We have identified putative transcription factor binding sites (TFBS) that have been shown to regulate the mammalian *DAX1* promoter transcription. Moreover, we have compared the promoter conservation of fish *DAX1* putative promoters to those described in mammalian and have detected a surprising conservation in promoter architecture, as we have identified different promoter frameworks composed of 3-5 transcription factor binding sites (TFBS) that are present in most of the *DAX1* promoters, within the same order and distances. In this chapter we have also identified putative gene networks that share this promoter architecture, allowing the identification of possible roles for *DAX1* gene in fish.

In **Chapter 5 and Chapter 6**, we have studied one of the networks identified in the *DAX1* promoter analysis described in chapter 4. Thus, in these two chapters we have studied the involvement of the *DAX1* in male gonad steroid production. In **Chapter 5**, we have studied the effect of a calmodulin inhibitor on the *in vitro* androgen production and in **Chapter 6** we have studied the effect of this inhibitor on steroid production, *in vivo*, and also its effect on *DAX1* and *DAX2* mRNA expression, as well as in other steroid enzymes, steroid receptors and other sex determining genes in male gonads.

In **Chapter 7**, the main results obtained in this thesis are highlighted and discussed.

CHAPTER 2

**Isolation, characterization and developmental expression of
DAX1 in the European seabass, *Dicentrarchus labrax*.**

With: Laurence A.M. Deloffre, Constantinos C. Mylonas, Deborah M Power and Adelino V M Canário. Reproductive Biology and Endocrinology (2007) 9: 19.

Abstract

DAX1 (*NR0B1*), a member of the nuclear receptors super family, has been shown to be involved in the genetic sex determination and in gonadal differentiation in several vertebrate species. In the aquaculture fish European sea bass, *Dicentrarchus labrax*, and in the generality of fish species, the mechanisms of sex determination and differentiation have not been elucidated. The present study aimed at characterizing the European sea bass *DAX1* gene and its developmental expression at the mRNA level. A full length European sea bass *DAX1* cDNA (*sbDAX1*) was isolated by screening a testis cDNA library. The structure of the *DAX1* gene was determined by PCR and Southern blot. Multiple sequence alignments and phylogenetic analysis were used to compare the translated *sbDAX1* product to that of other vertebrates. *SbDAX1* expression was analysed by Northern blot and relative RT-PCR in adult tissues. Developmental expression of mRNA levels was analysed in groups of larvae grown either at 15°C (feminizing temperature) or 21°C (masculinizing temperature) during the first 60 days, or two groups of fish selected for fast (mostly females) and slow growth (mostly males). The *sbDAX1* is expressed as a single transcript in testis and ovary encoding a predicted protein of 301 amino acids. A polyglutamine stretch of variable length in different *DAX1* proteins is present in the DNA binding domain. The *sbDAX1* gene is composed of two exons, separated by a single 283 bp intron with conserved splice sites in same region of the ligand binding domain as other *DAX1* genes. *sbDAX1* mRNA is not restricted to the BPG axis and is also detected in the gut, heart, gills, muscle and kidney. *SbDAX1* mRNA was detected as early as 4 days post hatching (dph) and expression was not affected by the incubation temperature. Throughout gonadal sex differentiation (60-300 dph) no dimorphic pattern of expression was observed. Altogether, we have shown that both *sbDAX1* gene and putative protein coding region is highly conserved and has a wide pattern of tissue expression. Although gene expression data suggests *sbDAX1* to be important for the development and differentiation of the gonads, it is apparently not sex specific.

2.1 Introduction

DAX1 (*NR0B1*) is a member of the nuclear receptors super family initially isolated from a 160 kb region in the human Xp21 chromosome, called *dosage-sensitive sex reversal (DSS)* region, that, when duplicated in the genome, is responsible for the *DSS* syndrome which causes male to female sex reversal in individuals with a normal *SRY* gene (Bardoni et al., 1994; Muscatelli et al., 1994). Mutations in this gene can give rise to a severe adrenal insufficiency, adrenal hypoplasia congenita (AHC) (Muscatelli et al., 1994), that is frequently accompanied by hypogonadotropic hypogonadism (HHG) (Muscatelli et al., 1994). Similarly, over expression of *DAX1* gene in certain mouse strains also leads to phenotypic sex reversal in males (Swain et al., 1998). Mice knockout models have also shown that *DAX1* expression in developing testicular tissue is essential for normal testicular development and for male fertility. *Dax1* deficient male mice present abnormal testicular morphology, and low sperm production and counts (Jeffs et al., 2001a; Jeffs et al., 2001b), whereas in female *Dax1* knockout mice, normal ovarian development and reproductive function is accomplished, although presenting a compromised endocrine function.

DAX1 is unusual in that it lacks the characteristic zinc-finger motif in the DNA-binding domain that is highly conserved in other members of the family (Muscatelli et al., 1994). In humans it encodes a protein of 470 amino acids, which N-terminus consists instead of three repeats of a unique 67 amino acids long cysteine-rich motif (Muscatelli et al., 1994). The number of repeats seems to vary throughout evolution, since in all homolog of non mammalian species studied so far only one repeat is present: chicken (Smith et al., 2000) alligator (Western et al., 2000), frog (Sugita et al., 2001) and in Nile tilapia (Wang et al., 2002). In the C-terminus, there is a nuclear receptor ligand-binding domain motif, even though no ligand is known to bind the protein (Zhang et al., 1998), and a bipartite transcriptional repression domain (Lalli et al., 1997). *DAX1* has been shown to be able to down regulate the transcription of several genes involved in the development and steroidogenic activity of both the adrenal and gonadal tissues, e.g. *StAR* (Lalli et al., 1997), *CYP17* (Hanley et al., 2001), *CYP19* (Wang et al., 2001), Müllerian inhibiting substance (*MIS*) (Nachtigal et al., 1998), estrogens receptors α and β (Zhang et al., 2000) and androgen receptor (Holter et al., 2002). Interestingly, another nuclear receptor, SF1, has also been shown to regulate most of the *DAX1* target genes within the steroidogenic pathway. In fact, it has been shown that there is a balance between repressor and inducer function of *DAX1* and SF1 that is critical for the regulation of key enzymes involved in steroid production (Hanley et al., 2001; Wang et al., 2001) Transcriptional repression by *DAX1* is thought to be mediated by both direct binding to gene promoters (e.g.

steroidogenic acute regulatory protein (StAR) Lalli et al., 1997; Lalli et al., 1998) and by interaction with different co-repressors: N-CoR (Crawford et al., 1998) and Alien (Altincicek et al., 2000). In addition, DAX1 is also suggested to play additional regulatory function in the post-transcriptional processes (Lalli et al., 2000). The European sea bass, *Dicentrarchus labrax*, is a gonochoristic teleost fish belonging to the family *Moronidae*, closely related to the Serranidae, a family composed of hermaphroditic species (Piferrer et al., 2005). This singularity has been suggested as being connected to its sex liability translated in the occurrence of intra-testicular oocytes in juveniles (Piferrer et al., 2005) and to a high degree of temperature-dependent sex differentiation (TSD) in farming conditions (Pavlidis et al., 2000; Koumoundouros et al., 2002; Saillant et al., 2002; Mylonas et al., 2005). Thus, incubating sea bass larvae at relatively high temperatures (above 17°C) strongly affects the sex ratio, increasing the proportion of males (Pavlidis et al., 2000; Koumoundouros et al., 2002; Saillant et al., 2002; Mylonas et al., 2005). The thermosensitive period for gonadal differentiation in sea bass corresponds to the first 60 days post fertilization (dpf) (Koumoundouros et al., 2002). The gonads become sensitive to steroids during a period of around 6 weeks centered at 100 dpf (Piferrer et al., 2005). The first gonad to differentiate is the ovary at 160 dpf (8 cm standard length, SL) followed by the testis, most males differentiating at 250 dpf (11.8 cm SL) (Piferrer et al., 2005). Size is a critical marker for the timing of sexual differentiation, so that larger larvae are more likely to develop as females while smaller larvae tend to develop as males (Blazquez et al., 1999). Thus, the identification of genes acting early in the sex determining cascade will facilitate understanding the mechanism involved in the TSD in this species and may provide markers for the early detection of sex. In teleost fish, including the sea bass, exogenous steroids given at the appropriate period of sex differentiation can lead to various degrees of sex change including sex reversal (Piferrer, 2001; Devlin and Nagahama, 2002). Thus natural and artificial estrogens feminize the gonads while non-aromatizable androgens and aromatase inhibitors have a masculinizing effect (Piferrer, 2001; Devlin and Nagahama, 2002). Furthermore, down regulation of *CYP19* transcripts has been associated to temperature-induced masculinization in Nile tilapia (D'cotta et al., 2001; Kwon et al., 2002) as well as with testis differentiation in reptiles with TSD (Jeyasuria and Place, 1998). Considering the above, the high conservation of sex differentiation pathway in vertebrates (Western et al., 2000) and the repressive action of DAX1 on *CYP19* (Wang et al., 2001) we hypothesize that *DAX1* expression is directly or indirectly up regulated by increasing temperatures. As a result *CYP19* would be down regulated and masculinization would follow.

In this chapter we investigate the possible role of *DAX1* in sex differentiation in European sea bass. We have cloned and characterized a cDNA for a European sea bass

DAX1 - homolog, determined the gene structure and analyzed its mRNA expression pattern in early gonadal development in fish cultured at normal and masculinizing temperatures and during gonadal differentiation in female and male biased populations.

2.2 Methods

2.2.1 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. The fish utilized to investigate gene expression during the first 60 dph (days post hatching) came from an experiment in which sea bass eggs were incubated at 15°C or 21°C until they reached a total length of 18 mm (48 and 66 dph, respectively). Thereafter both groups were maintained at natural temperature (ranging between 18 to 22°C) until sex determination was complete (300 dph) and gonadal sex was determined histologically. Rearing conditions were similar to those described by (Mylonas et al., 2005). Treatment resulted in 75% and 22% females in the 15°C group and 21°C groups respectively. Samples for gene expression analysis were taken at 4, 18, 32, 46 and 60 dph to analyze gene expression. At 4 dph samples were 6 pools of 3 larvae per sample, at other times consisted of 6 individual larvae.

The fish to analyze 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 dph 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. The percentage of females in the smaller fish population decreased from 50% as a result of the 120 days grading, 43% after the 166 days grading and 30.2% following 200 days. In the larger population during the same period the % females were 91%, 92%

and 96.5%. Samples (n=3-8 per point) to analyze gene expression were taken at 56 dph (whole larvae), at 100, 150, 200, 250 dph (body trunks) and at 300 dph (gonad).

For sample collection, fish were killed with an overdose of 2-phenoxyethanol (1:5000, Sigma-Aldrich, Madrid, Spain). Whole larvae or dissected tissues in older fish were immediately frozen in liquid nitrogen and stored at -80 °C until used (normally within 3 months).

2.2.2 RNA isolation and Reverse transcription

Total RNA was isolated from larvae, gonads and other various tissues of adult sea bass (1.5 years old) with TRI reagent (Sigma-Aldrich, Madrid, Spain) according to the manufacturer's instructions. Total RNA (3-5 µg) was reverse-transcribed in 40µl at 37°C for 60 min., using 200 units of superscript, 0.25 mM dNTPs, 0.2 units of RNase inhibitor and 3 µg random-hexamers. Poly (A)⁺ RNA was isolated from total RNA using mRNA purification kit from Amersham Pharmacia Biosciences (Lisbon, Portugal).

2.2.3 Isolation of the sea bass *DAX1* full length cDNA

A pair of oligo nucleotide primers (Tildax, Table 1) was designed based on the sequence of tilapia *DAX1* (Wang *et al.*, 2002) and used in reverse transcriptase-polymerase chain reaction (RT-PCR) to isolate a 587bp fragment of *DAX1* from testis cDNA. RT-PCR amplification was carried out in 25 µl reactions containing 1µl of cDNA, 2.5µl of 10x Buffer (Promega), 2.0 mM MgCl₂, 0.04 dNTPs, 0.4 µM sense and anti sense primers, and 0.5 units of taq Polimerase (Promega) (see also Table 1). Products were separated and purified from an agarose gel (1%) with the GFX PCR DNA and Gel Purification Kit (Amersham Biosciences), cloned in pGem Teasy vector (Promega, Madison, USA) and sequenced. The nucleotide sequence of the PCR product had 90% similarity with that of tilapia *DAX1* cDNA. The sea bass *DAX1* fragment was radio labeled using a random labeling kit (Rediprime II, Amersham-Pharmacia) and used to screen a sea bass testis cDNA library constructed in Lambda Zap using the UNIZAP vector (Stratagene). After screening 4x10⁵ recombinants at moderate stringency (in Church-Gilbert buffer at 55°C), four independent clones were auto excised in p Bluescript phagemid using helper phage. Two clones, 2LD and 5LD, each containing a 1373 bp insert, were isolated and completely sequenced by primer walking. The two clones were identical.

Table 1- Primers and RT-PCR conditions for *DAX1* isolation and gene expression analysis. Primers Ti1Dax1, sbDAX1 and 18S were used to amplify cDNA in relative semi-quantitative RT-PCR. Primers F1-F3 sense primers were used in various combinations with anti sense primers R1-R4 in the determination of gene structure.

Primer	Primer sequences (5'-3')	Thermocycles
Ti1Dax1 Fw Ti1Dax1Rv	CTGGTGAAGACGGTGCGGTTTCGT CACTATGGACCCGTGACCTACTT	35 cycles: 94°C 1 min 57°C 1 min 72°C 1 min
sbDax1 Fw sbDax1 Rv	CTGGGGGGTTCTGGTGAAG CGGTCTCCGTGGTCTCAAAGTC	32 cycles: 94°C 1 min 59°C 1 min 72°C 1 min
18S Fw 18S Rv	TCAAGAACGAAAGTCGGAGG GGACATCTAAGGGCATCACA	18 cycles: 94°C 1 min 59°C 1 min 72°C 1 min
F1 F2 F3	CTGGCGAAAACCTTTCGATTCCT CTCGGCGGTTCTGGTGAAG GTGGAGCCCAGCATGCTGCAGC	
R1 R2 R3 R4	CGGTCTCCGTGGTCTCAAAGTC AGCCTCCCTGTGAAGTGTCTGG CTTCCCGTCACCGGTCTGAAGAAG GCCAATGACCGGTTTGAAGAAG	

2.2.4 Multiple sequence alignment and phylogenetic analysis

The accession numbers of the *DAX1* sequences used in the alignments and/or in the phylogenetic analysis were from tilapia (AAN17672), medaka (ABQ 09274), sea bass (CAG17628), tetradon (CAG05777), zebrafish (AAY85268), chicken (AF202991), human (NP000466), mouse (CAM21707), rat (NP445769) and the *DAX1* from fugu was mapped to Fugu Consortium Scaffold S000386 (<http://fugu.biology.qmul.ac.uk/>). The accession numbers of the *DAX2* sequences used in the alignments and phylogenetic analysis were from tilapia (ABB88831) and the *DAX2* from fugu was mapped to Fugu Consortium Scaffold S002663 (<http://fugu.biology.qmul.ac.uk/>). The accession numbers of the *SHP* sequences used in the alignments and phylogenetic analysis were from tilapia (AAN17673), tetradon (CAG00032), zebrafish (AAH580691 and AAY85285), chicken (AAW33564), human (NP068804), mouse (NP035980), rat (NP476474), medaka (ENSORL00000005570), and the fugu SHP proteins [(designated in this work SHP1 (SINFRUT00000151053) and SHP2 (SINFRUT00000151053)]. Alignments were performed using the ClustalW. Phylogenetic analysis was performed using Neighbour joining method with 1000 bootstrap sampling (software: MEGA 3.1, 1993-2005).

2.2.5 Southern and northern blot analysis

High molecular weight DNA was isolated from liver of 3 females and 3 males, using a standard extraction method with phenol: chloroform. Ten µg were digested with 45 U ECOR1 (Takara Biomedicals, Shiga, Japan) at 37°C over night and the fragmented DNA was separated on agarose gel (0.7%), the gel was treated with a 0.250 M HCl, denatured in 1.5 M NaCl/0.5 M NaOH and neutralized in 1.5 M NaCl/0.5 M Tris Base, pH=8. The DNA was transferred in 20 x SSC by capillarity onto a nylon membrane (Hybond XL, Amersham Pharmacia). The membrane was hybridized with (α-³²P) CTP labelled 587 bp *DAX1* cDNA fragment in Perfect Hyb solution (Sigma), according to the manufacturer's instructions. Probe labeling was by random priming (Rediprime II, Amersham-Pharmacia).

For northern blot, total mRNA from 3 testes and 3 ovaries was isolated from fish with 2.5 years old as previously described. The RNA (5 µg for testis and 10 µg from females) was heat denatured for 5 min (65°C), run on a 1% denaturing formaldehyde gel, and transferred onto a nylon membrane (Hybond XL, Amersham Pharmacia). Hybridization with a *DAX1* probe covering the full ORF (Open reading frame) followed the same procedure as described for Southern blotting analysis.

2.2.6 Gene structure

Different sets of forward (F1-F3) and reverse (R1-R4) primers were designed according to the *sbDAX1* full length sequence, and used in all possible combinations, to amplify the intron sequences present within the *DAX1* gene from liver genomic DNA, isolated as described.

2.2.7 Semi-quantitative PCR analysis

In all RT-PCR reactions, 1µl of cDNA was amplified in a reaction of 25 µl containing 2.5 µl of 10x Buffer (Promega), 2.0 mM MgCl₂, 0.04 dNTPs, 0.4 µM of *DAX1* specific primers (*sbDAX1* Table 1), and 0.5units of Taq polymerase (Promega). The thermocycles were optimized in order to obtain a single product within the linear range of RT-PCR amplification. For all samples RT-PCR amplification of 18S rRNA was used as reference in the same conditions as above except for 1.0 mM MgCl₂ and 0.4 µM 18S primers (18S Fw/18S Rv, Table 1). The *DAX1* and 18S RT-PCR reaction products were separated on agarose gel (0.7%), denatured (1.5M NaCl/0.5 M NaOH), neutralized (1.5M

NaCl/0.5 M Tris Base, pH=8) and transferred onto nylon membranes (Hybond XL, Amersham Pharmacia) in 6x SSC solution by a standard capillarity method. The membranes were hybridized with (α -³²P) CTP radio labeled probes (161 bp *DAX1* and 450 bp 18S) in Church Gilbert buffer (65°C), and subjected to stringent washing (1x SSC/0.1% SDS and 0.1x SSC/0.1% SDS) at 65°C. The membrane signals were quantified by phosphor-imaging (GS-505 Molecular Imager System, Biorad) and expressed as the ratio of amplified target to that of 18 S ribosomal RNA.

2.2.8 Statistics

The ratios of *DAX1* to 18S were log transformed and the effects of temperature or grading and age were analysed by two-way Analysis of Variance followed by the Tukey multiple comparison test with a significance level of 5%. Unless otherwise stated the results are expressed as mean $\pm$ standard error of the mean.

2.3 Results

2.3.1 Cloning of sea bass *DAX1* and sequence analysis

A 1373 bp *DAX1* clone (named sb*DAX1*) was obtained from screening approximately 4×10^5 recombinants of a testis cDNA library. The 5' untranslated region (UTR) of 116 bp ends with a partially conserved Kozak sequence (CCATGGC) signaling the beginning of an open reading frame (ORF) encoding 301 amino acids (Appendix 1). The 3'UTR is approximately 350 bp, and contains a putative poly adenylation signal at nucleotides 1334-1340 and a poly (A)⁺ tail. The sequence has been deposited to Embl (accession number AJ633646). PCR of sea bass genomic DNA resulted in the amplification of several DNA fragments covering the coding region of sb*DAX1*, but only the primer combination F3-R4 contained a 283 bp intron, located at amino acid positions 221-222, within the LBD (Fig. 1). This intron is located in the exact position as all other *DAX1* genes described so far, confirming that the structure of this gene is maintained throughout all taxa.

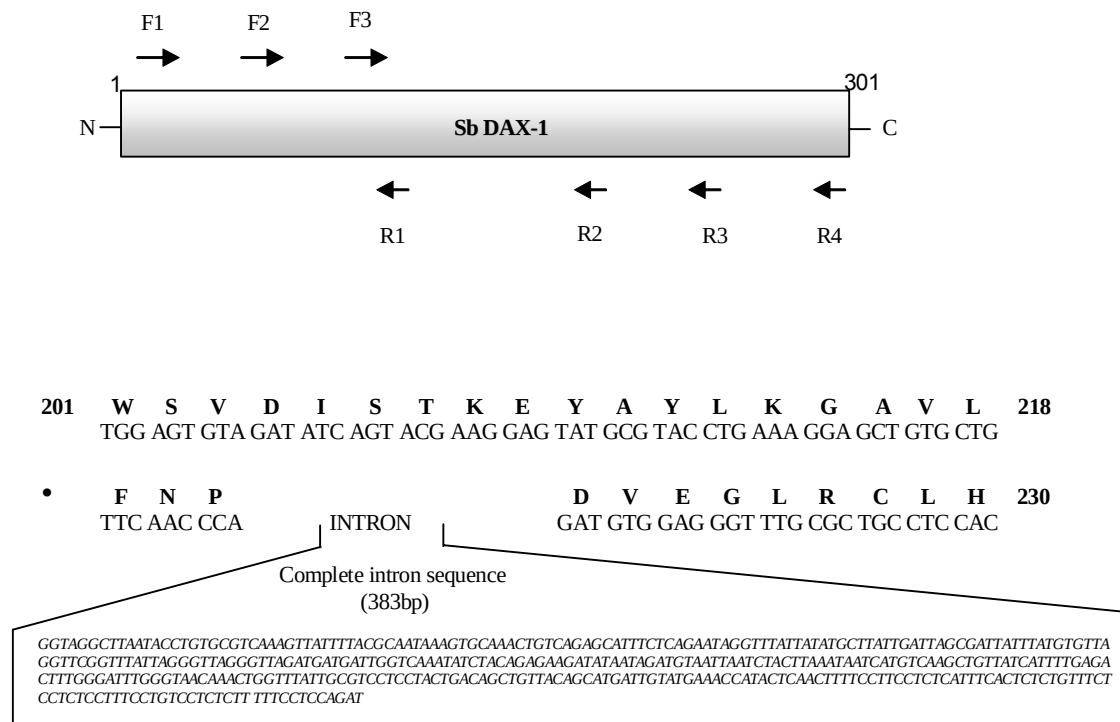


Figure 1– The European sea bass DAX1 gene structure. Different sets of forward (F1-F3) and reverse (R1-R4) primers were used to amplify the DAX1 gene from genomic DNA. PCR amplification yielded a single 383 bp intron sequence, positioned between amino acids 221 and 222 within the LBD.

Multiple sequence alignment revealed that the predicted sbDAX1 protein shared highest overall sequence identity and similarity to fugu followed by, in descending order, tilapia, chicken, frog, alligator and human (Table 2). While a high level of conservation was found for the carboxy terminal region (higher than 50%), the amino terminus was relatively more poorly conserved.

Table 2- Comparison of DAX1 protein sequence identity and similarity between European sea bass and other vertebrates. Sequence identity and similarity (parentheses) of the DAX1 DNA binding domain (N-terminal), carboxy-terminal region (C-terminal) and whole protein (DAX1 protein) from different vertebrates (Species).

Species	N-Terminal (%)	C-Terminal (%)	DAX1 protein (%)
Fugu	80 (89)	90 (94)	86 (92)
Tilapia	69 (78)	94 (96)	85 (89)
Frog	33 (44)	56 (73)	48 (62)
Alligator	19 (26)	59 (73)	43 (54)
Chicken	32 (39)	62 (78)	51 (62)
Human	11 (18)	49 (66)	25 (34)

The main reasons for the poor conservation of the putative DNA binding domain is the much longer N-terminal human protein compared to all vertebrates and the presence of region of variable length and amino acid sequence adjacent to a generally conserved nuclear LXXLL-like motif (NR box3) (Fig. 2). In the sea bass DAX1 this region corresponds to a poly glutamine stretch composed of 13 Glu (amino acid positions 36-48) and two extra glutamine repeats composed of 4 and 2 Glu (amino acid positions 51-54, 57-58). As with all the non-mammal vertebrates the sbDAX1 protein lacked NR box1 and NR box2 of the three LXXLL-like repeats present in the human DAX1 DNA binding domain. Similarly, sea bass, like chicken, frog, alligator, tilapia, and fugu has only a partially conserved copy of NR box3 (-NSILYNILKS-) at amino acids positions 17-27.

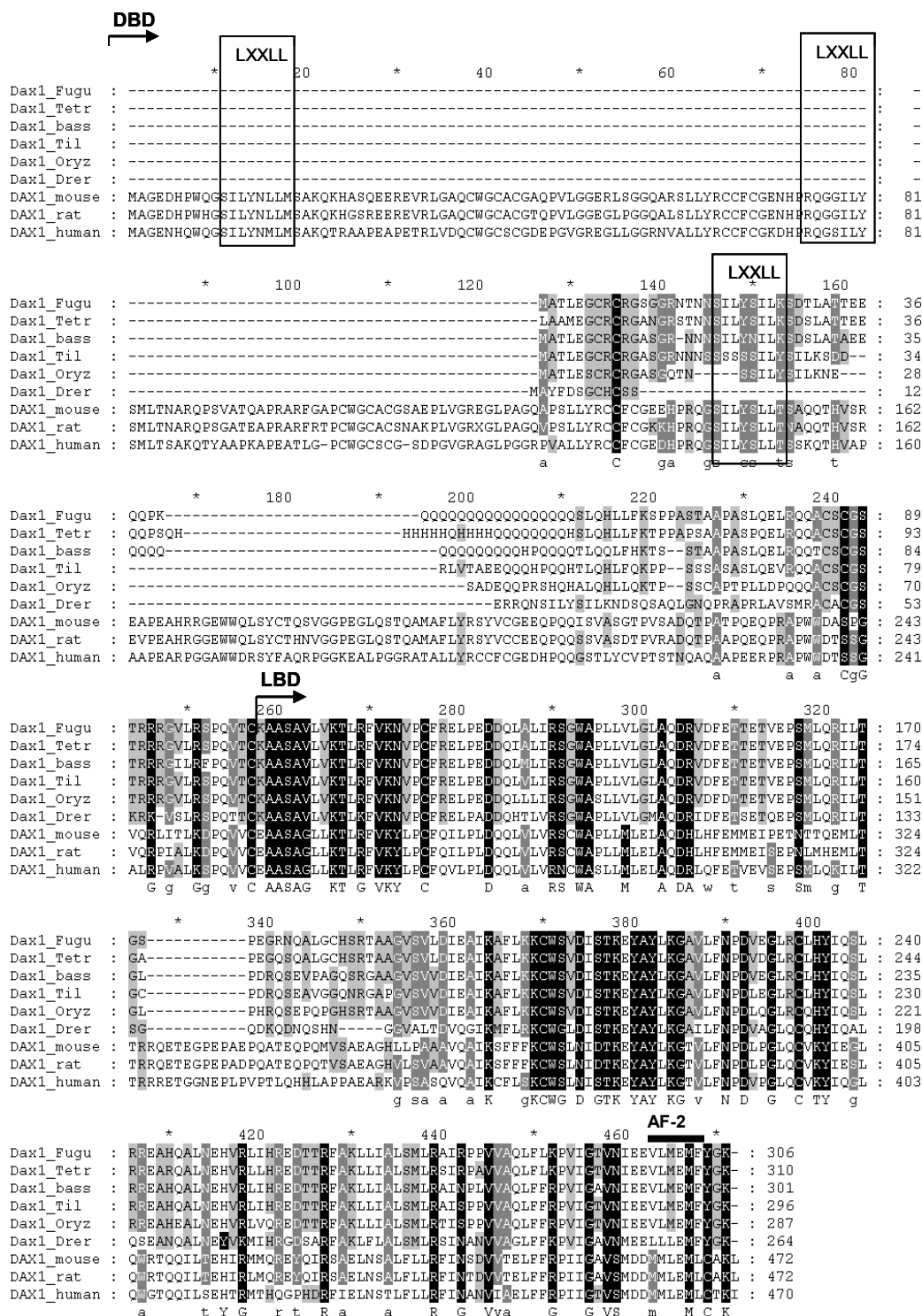


Figure 2 – Multiple sequence alignment of DAX1 protein. Identical and similar amino acids are marked with asterisks and dots, respectively. The putative DNA-binding domain (DBD) and ligand binding domain (LBD) are indicated. The conserved LXXLL-like motif and the AF-2 core, in the DBD and LBD, respectively, are indicated inside boxes.

Phylogenetic analysis shows that fish DAX1 cluster together with other fish DAX1 and separate from the tetrapods (Fig. 3).

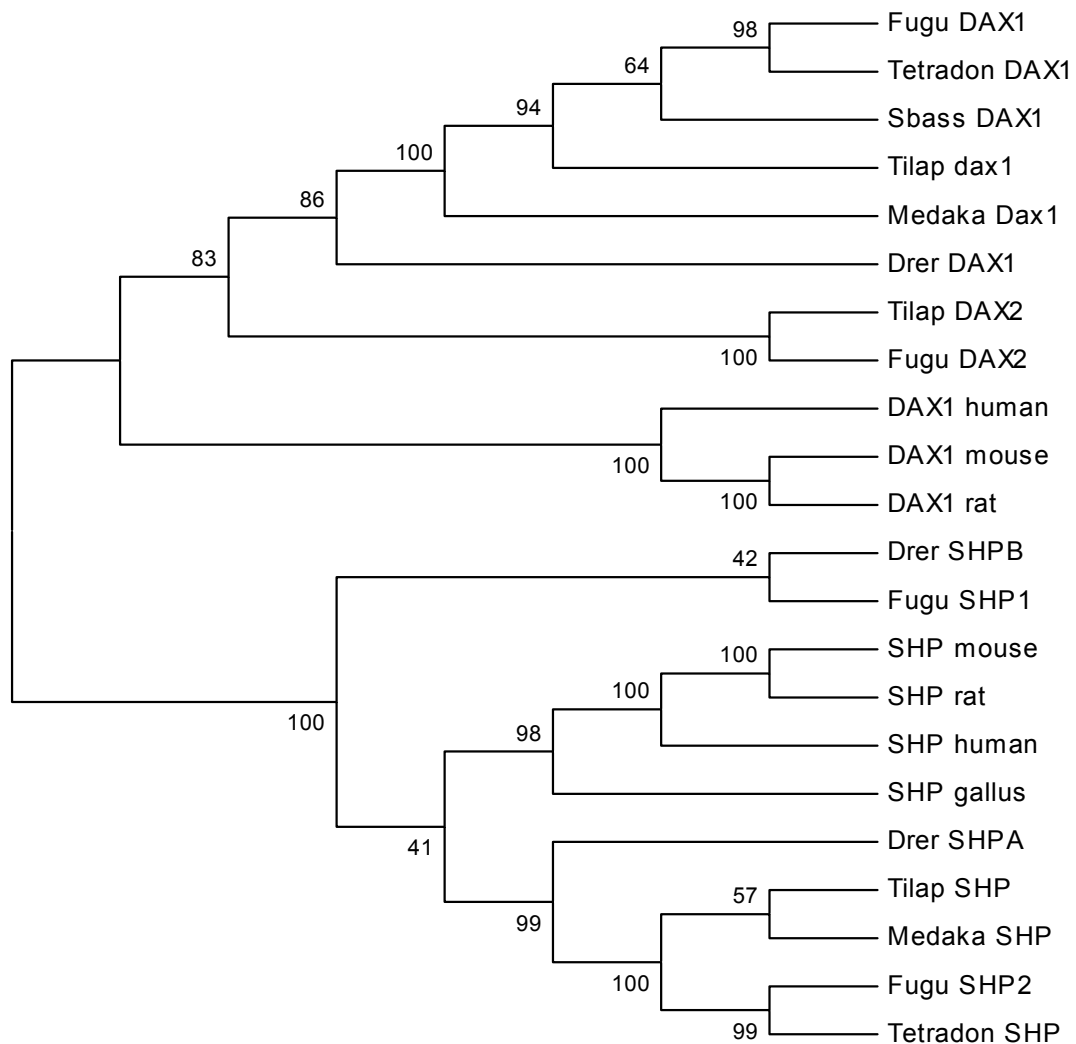


Figure 3– Phylogenetic tree of DAX1 protein with Nr0B family members. Consensus tree obtained by Neighbour joining analysis with corresponding bootstrap values from sampling 1000 trees. Sequence's accession numbers are documented in the Methods section. Species's abbreviations used in this tree: Tilap (tilapia), Sbass (sea bass) and Drer (zebrafish). Short heterodimer partner (SHP) sequences were used to root the tree.

Southern blot analysis showed a single band of similar size (~5.2 Kb) in DNA from both sexes (Figure 4a). Northern blot analysis yielded a single transcript of ~1.3 kb in both ovary and testis (Figure 4b).

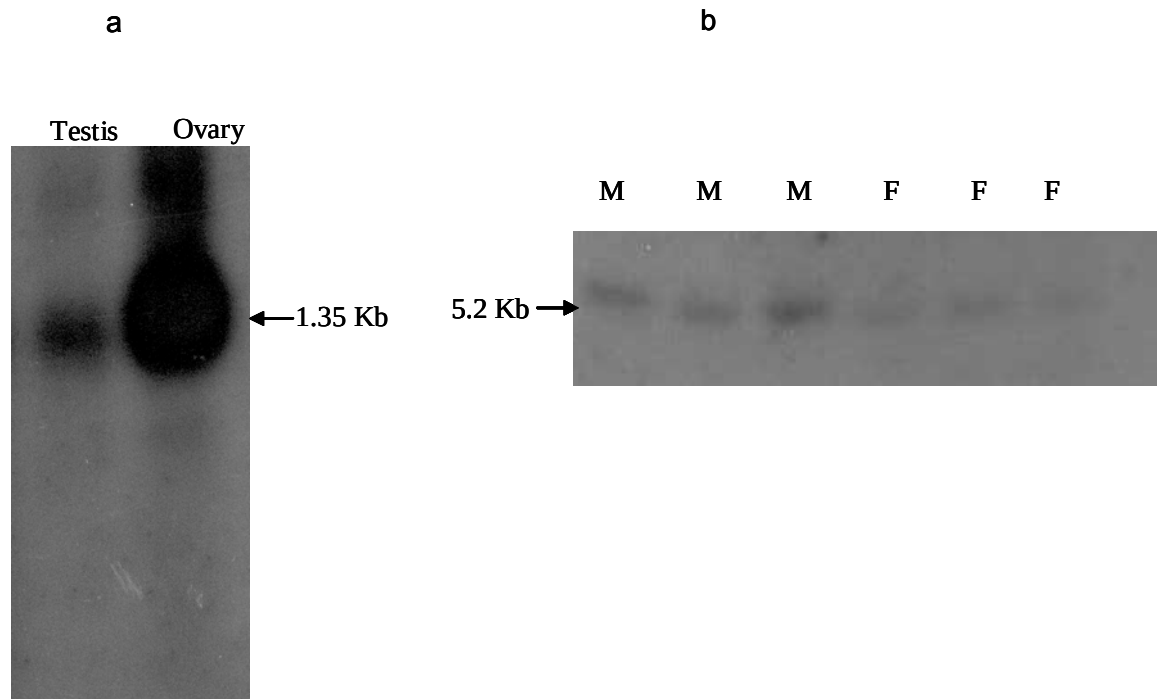


Figure 4– Northern blot and Southern blot hybridizations with the *DAX1* gene. a) Total RNA from testis (5 µg) or ovary (10 µg) was run on 1% denaturing formaldehyde gel, transferred onto a nylon membrane and hybridized with the sea bass *DAX1* full length cDNA. B) Genomic DNA of three males (M) and three females (F) was digested with *EcoRI* and hybridized with the sea bass *DAX1* gene.

2.3.2 Tissue distribution of *DAX1* mRNA expression

The pattern of sb*DAX1* expression in female and male tissues was determined by relative RT-PCR analysis (Fig. 5). sb*DAX1* is widely expressed with a relatively balanced expression in the two sexes in brain, pituitary and gonads, but an apparent male dominance in a range of other tissues, including head and trunk kidney, digestive tract, heart, liver, muscle and gills.

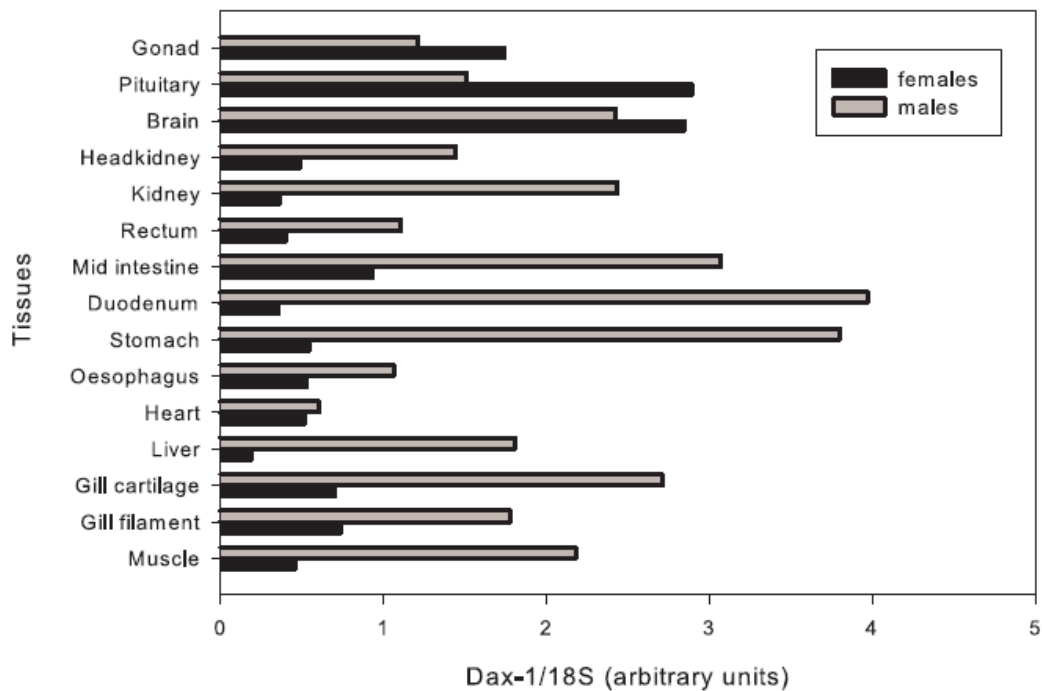


Figure 5 – DAX1 gene expression in different tissues from adult European sea bass. RT-PCR of DAX1 relative to 18 S in pools of tissues from three females or three males.

2.3.3 *DAX1* mRNA expression during early development

The expression pattern of *sbDAX1* during the temperature sensitive period for sex differentiation was determined by relative RT-PCR in larvae from 4 dph to 60 dph. At 15°C *sbDAX1* expression was always detected and at similar levels in larvae grown at 21°C (Fig. 6). The levels of *sbDAX1* expression were significantly lower at 18 days, compared to any of the other points sampled ($p < 0.05$).

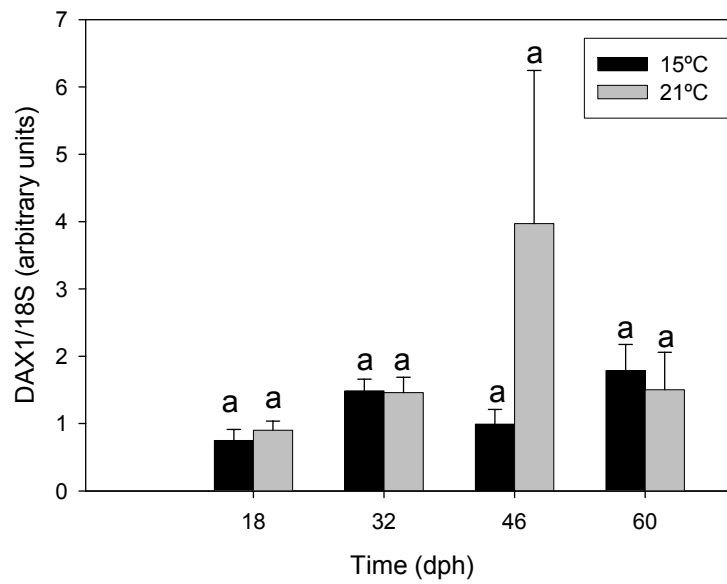


Figure 6– *DAX1* gene expression during the temperature sensitive sex determining period in European sea bass. RT-PCR of *DAX1* relative to 18S in sea bass larvae grown at 15°C or at 21°C during the thermo sensitive period for sex determination ($n=6$ at each sampling point). Larvae grown at either 15°C or 21°C resulted in 77.5% and 28% females, respectively. Different letters denote statistical differences between the groups when $p<0.05$.

The expression pattern of *sbDAX1* between 56 dph and 300 dph, encompassing gonadal differentiation, was determined by relative RT-PCR (Fig. 7). *sbDAX1* expression was detected in all samples, and no significant differences were found between larger and smaller fish. Significantly lower *DAX1* expression was detected at 150 days ($p<0.005$).

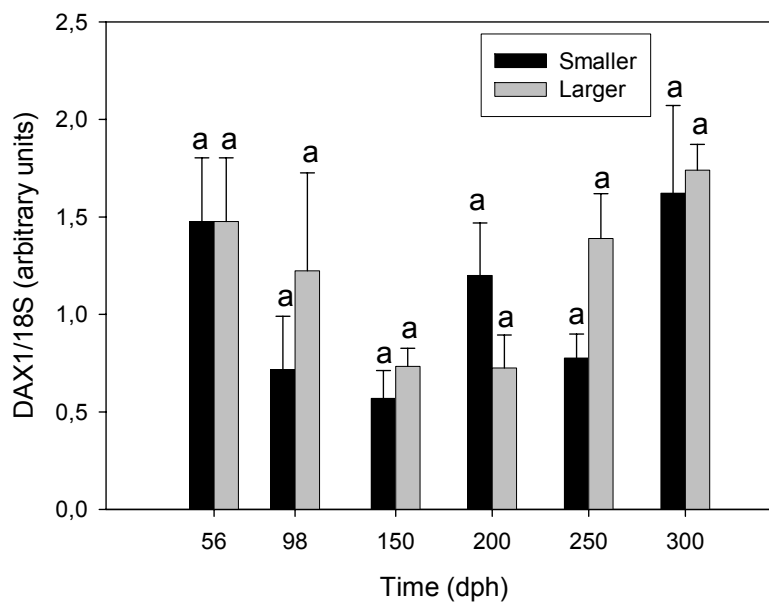


Figure 8– *DAX1* gene expression during the gonadal differentiation in size graded sea bass. RT-PCR of *DAX1* relative to 18S in whole larvae (56 days), body trunks (98, 150, 200 and 250 days) or gonads (300 days) during the period of sex differentiation ($n=3-8$). Larvae were repeatedly graded according to size. Smaller fish developed into a majority of males (35.4% females) and larger fish developed into a majority of females (96.0% females). Different letters denote statistical differences between the groups when $p<0.05$.

2.4 Discussion

In this study we have identified a full length cDNA clone of a *DAX1* homolog gene in the European sea bass. The sea bass *DAX1* cDNA clone encodes a 301 amino acids protein that shared highest overall sequence identity and similarity to other *DAX1* homologs in fish (fugu, tilapia, zebrafish and rainbow trout) followed by birds, reptiles and mammalian. As all *DAX1* genes described so far, the sea bass *DAX1* gene lacks the characteristic zinc-finger motif in the DNA-binding domain (DBD) that is highly conserved in other nuclear receptors (Muscatelli et al., 1994) but, as in other non mammalian species, teleost fish [Nile tilapia (Wang et al., 2002), amphibians (Sugita et al., 2001), reptiles (Western et al., 2000), and birds (Smith et al., 2000)], only one of the three LXXLL-like motifs present in the DNA-binding domain of the human *DAX1* aligns at the same positions of the putative DBD (NR box 2 in Fig 3). It has been documented that these LXXLL-like motifs are implicated in nuclear receptor binding, namely in interacting with SF1 (Ito et al., 1997; Kawajiri et al., 2003), estrogen receptor (Zhang et al., 2000),

androgen receptor (Holter et al., 2002) and progesterone receptor (AgoulNIK et al., 2003). Moreover it has been shown that all LXXLL-like motifs in DAX1 are able to interact with other nuclear receptors, although with different binding affinities (Zhang et al., 2000). Thus, it appears that DAX1 from sea bass and from other non mammal vertebrates is able to interact with nuclear receptors through the conserved NR box 2. Intriguingly, however, the sea bass and fugu first LXXLL-like motif is followed by a polyglutamine (polyQ) stretch. In sea bass, the polyQ stretch is composed of 13 glutamines followed by two extra glutamine repeats composed of 4 and 2 glutamines. In the Fugu DAX1 the polyQ stretch is also composed of 17 glutamines but it is uninterrupted. In contrast, Nile tilapia contains only seven scattered glutamines within this region, rainbow trout 6 and zebrafish only 3 (Zhao et al., 2006). Glutamine is the most common repeated amino acid in eukaryotic proteins, and it has been postulated that the introduction of such repeats is a general evolutionary mechanism for adding new amino acid sequence (Kahlem et al., 1996). PolyQ stretches are also functionally associated to human disorders and it has been shown that the polyQ stretch length is correlated to the appearance of polyQ peptides in inclusion of highly insoluble aggregates in the nucleus or cytoplasm (for reviews see Michalik and Van Broeckhoven, 2003). Thus, aggregation can compromise function by sequestering the protein away from their usual localization, but also because these aggregates are able to interact with several transcription factors harbouring short glutamine repeats [e.g. cAMP response element binding protein (CBP) and TATA-binding protein (TBP), (Perez et al., 1998; Kazantsev et al., 1999)] via glutamine-glutamine interactions, or with other interaction partners (Chai et al., 2001). Moreover, it has been shown that expanded polyQ tracts in genes involved in male sexual differentiation in tetrapods, result in abnormal sex differentiation. Breeding the Y chromosome in certain *Mus musculus domesticus* strains onto the inbred laboratory mouse strain, C57BL/6J (B6), results in hermaphroditic progeny, with different degrees of sex reversal, as a consequence of specific changes in the predicted polyQ amino acid sequence at this site (Coward et al., 1994). Also, extended polyQ tracts in the transactivation domain of the androgen receptor (AR) are associated to increased risk of defective spermatogenesis and under masculinization, depending on the genetic background (reviewed in Dakouane-Giudicelli et al., 2006). Considering the above and although, the data available is rather small, it is tempting to speculate that the length of the glutamine stretch in DAX1 from fishes could be positively correlated to susceptibility to masculinization by temperature. While in European sea bass, with a large number of polyQ, temperatures above 17°C (2-4°C above the natural spawning temperature) cause masculinization (Blazquez et al., 1999), in *Onchorynchus* species (closely related to rainbow trout), zebrafish and tilapia (with a small number of polyQ), temperatures close to the lethal upper limit are required

for some degree of masculinization to occur (Baroiller et al., 1995; Craig et al., 1996; Abucay et al., 1999; Uchida et al., 2004). In this hypothesis, the formation of DAX1 aggregates could limit the availability of active DAX1 protein and reduce its inhibitory action on masculinizing factors, such as SOX9 (Zhang et al., 2000; Tremblay and Viger, 2001; Holter et al., 2002). However, this does not explain the susceptibility to temperature in tetrapods, which appear to be highly dependent on kinetic properties of the aromatase enzyme (Pieau et al., 1999).

The C-terminal of DAX1, presents a high degree of conservation in all species studied so far. To date, all mutations in adrenal hypoplasia congenita localize to the C-terminal part of the gene, suggesting that this region represents the critical domain for DAX1 function (Achermann et al., 2001a). This domain has been shown to be responsible for transcriptional silencing activity in mammal species in a similar way to a restricted subset of other members of the nuclear receptor super family: thyroid hormone (TR), the related oncogene product v-erbA, retinoic acid receptor (RAR), and the chicken ovalbumin upstream promoter transcription factor (COUP-TF) (Lalli et al., 1997). The silencing activity of DAX1 is possible due to a bipartite domain in the C-terminal (Lalli et al., 1997) that corresponds to the helix 3 and helices 11-12, according to the DAX1 protein structure prediction when aligning DAX1 with RAR, TR and v-erbA sequences (Lalli et al., 1997; Altincicek et al., 2000). The fact that all DAX1 sequences are highly conserved within these regions, suggests a common mechanism across the vertebrates. This is further supported by the presence of a highly conserved AF-2 core sequence at the C-terminal end.

In the Southern blot a single band was detected in males and females indicating that *DAX1* is the product of a single gene and that it is not sex-specific. Moreover, genomic DNA amplification of the *DAX1* gene in sea bass from both sexes has also shown that this gene has a two exon structure, separated by a single intron with 383 bp and conserved splice sites located at the same region of the ligand binding domain as all other DAX1 genes described so far. These results confirm what had been observed in the Southern blot analysis and thus emphasize that in this species the *DAX1* gene is transcribed from a single gene. Northern blot analysis detected a ~1.3 kb transcript in gonads of mature (2-year old) fish of both sexes, similar to that isolated in the testis cDNA library. These results are similar to those obtained in tilapia (Wang et al., 2002), chicken (Smith et al., 2000) and alligator (Western et al., 2000). In contrast, no expression was detected in ovary or testis of adult frogs, which was suggested to be due to the fact that after metamorphosis is complete only spermatogonia could be found in testis, while in younger animals, Leydig cells could be detected (Sugita et al., 2001). The *DAX1* mRNA was not confined to reproductive tissues but instead it was present in all tissues analyzed.

Although higher levels of *DAX1* were apparent in brain, pituitary and gonads of female sea bass, the reverse was true for head kidney, kidney, digestive tract, liver, heart, gills and muscle. The same broad expression in tissues and differences in expression levels between male and female tissues have also been documented for tilapia, although male tilapia brain expressed higher levels of *DAX1* (Wang et al., 2002). In frog and mouse *DAX1* is also expressed in several tissues other than the brain-pituitary-gonadal/adrenal axis (Bae et al., 1996; Sugita et al., 2001). ER α , another nuclear receptor, was also higher in female compared to male sea bass liver (Halm et al., 2004). Nonetheless, the biological significance of this dimorphic pattern in non reproductive tissues is hard to interpret at present.

In the sea bass, *DAX1* mRNA was detected as early as day 4 post hatching, well before the period of sex differentiation (Piferrer et al., 2005), in accordance with similar observations in other vertebrates (Smith et al., 2000; Sugita et al., 2001; Morrish and Sinclair, 2002; Wang et al., 2002), including zebrafish (Zhao et al., 2006) and with its essential role in steroidogenic enzyme and interrenal/adrenal development (Parker et al., 1999; Zhao et al., 2006). In fact, recent observations in *Danio rerio* have underlined the role of *DAX1* in the interrenal development where it has been found that there is a weak and transient expression of this gene at approximately 31 hours post fertilization (hpf) in the interrenal, and also that disruption of *DAX1* function by morpholino oligonucleotides resulted in the down regulation of steroidogenic enzymes STAR and CYP11A in this tissue (Zhao et al., 2006). The expression of *DAX1*, however, did not follow a dimorphic expression pattern at any time during sea bass development, including the sex differentiation period. At 150 days (gonadogenesis) there was an apparent decrease in expression, although the tissue analyzed was not exclusively gonads (body trunks). Coincidentally, this is also the period of highest sensitivity to androgen treatments (Blazquez et al., 2001) and when there is a dimorphic pattern of androgen receptor (AR) α and 11 β -hydroxylase mRNA expression with higher levels in the group dominated by males (Blazquez and Piferrer, 2005; Socorro et al., 2007). Considering this temporal pattern and also the documented regulation of *DAX1* on AR in mammalian species, the *DAX1* could be a candidate gene to regulate the effects of androgens on gonadal differentiation. In human, *DAX1* protein is able to interact with the AR protein through direct interaction of the *DAX1* N-terminal repeat domain with the AR C-terminal ligand binding and activation domain (Holter et al., 2002). By blocking the interaction of the N-terminal and C-terminal of the AR, the *DAX1* will also inhibit the ligand-dependent transcriptional activation of the AR, and thus influence the regulation of androgen-dependent gene transcription in the male reproductive system (Holter et al., 2002). Despite the early expression of *DAX1* in sea bass larvae, we could not detect any

influence of temperature on *DAX1* expression during the thermo sensitive period, although at 46 dph we could detect higher levels at 21°C than at 15°C. These results are possibly due to the fact that at this stage (4-60 dph) there is no gonadal development and thus, the expression levels obtained are from whole body larvae. In alligators, an increase in gonadal *DAX1* expression was detected during the temperature- sensitive period, although, as found in sea bass, *DAX1* expression was not sexually dimorphic during gonadogenesis (Western et al., 2000). Although no dimorphic pattern could be detected at the mRNA level during both thermosensitive period and gonadal differentiation period, it is possible that at the protein level, *DAX1* is interacting or regulating other proteins that are involved in determining the sexes. Thus, future studies on protein interactions with the sb*DAX1* protein should give us a better insight on the role of this protein in the process of gonadal differentiation. Also, immunocytochemistry or in situ hybridization should help to clarify the early pattern of gonadal *DAX1* expression.

In conclusion, we have isolated a full length cDNA from sea bass that has highest identity to other teleost fish *DAX1*. The mRNA expression of sb*DAX1* shows a dimorphic pattern of expression as higher levels are detected in the female gonads, brain and pituitary than in males, although out of this axis this gene is substantially more expressed in tissues from males than from females. Sea bass *DAX1* is expressed throughout ontogeny from day 4 post hatching until the end of gonadal differentiation (300 dph), although no dimorphic pattern is observed between male biased and female biased populations, suggesting that *DAX1* is important for gonadal differentiation but is not sex specific. Overall, no significant effect of temperature was found in *DAX1* expression throughout the temperature sensitive sex determining period.

APPENDIX

GGCACGAGGGCTATCAGGGGCTAAGAAAACAGCGCTACCTCTGTCACTGACCTCCGGCGCTTCAAAGAGAC	71
AGCGGGACGCGCTGAGCTCCATCCCATGTAGAGCGGCCAGCGGCC	6
ATG GCC ACG CTG GAG GGC	134
C R C R G A S G R N N N S I L Y N I	24
TGC CGC TGT CGG GGT GCC AGC GGC CGA AAC AAT AAC AGC ATC CTC TAC AAC ATT	188
L K S D S L A T A E E Q Q Q Q Q Q Q	42
TTG AAG AGT GAC AGC CTT GCG ACT GCC GAG GAG CAA CAA CAA CAA CAA CAA CAA	242
Q Q Q Q Q Q H P Q Q Q Q T L Q Q L F	60
CAA CAA CAA CAA CAA CAA CAT CCC CAA CAA CAA CAA ACC CTG CAA CAA TTG TTT	296
H K T S S T A A P A S L Q E L R Q Q	78
CAC AAG ACC TCC TCC ACC GCC GCC CCA GCC TCT CTG CAG GAG CTC CGG CAG CAG	350
T C S C G S T R R R G I L R F P Q V	96
ACT TGC TCC TGC GGC TCG ACC CGG CGC AGA GGT ATC CTC CGC TTC CCA CAG GTG	404
T C K A A S A V L V K T L R F V K N	114
ACG TGC AAA GCC GCC TCG GCG GTT CTG GTG AAG ACG CTG CGG TTC GTG AAA AAC	458
V P C F R E L P E D D Q L M L I R S	132
GTC CCC TGT TTT CGC GAG CTG CCG GAG GAC GAC CAG CTG ATG TTG ATC CGG AGC	512
G W A P L L V L G L A Q D R V D F E	150
GGC TGG GCA CCC TTG CTC GTG CTG GGA CTC GCA CAA GAC CGA GTG GAC TTT GAG	566
T T E T V E P S M L Q R I L T G L P	168
ACC ACG GAG ACC GTG GAG CCC AGC ATG CTG CAG CGC ATC CTC ACG GGT TTA CCA	620
D R Q S E V P A G Q S R G A A G V S	186
GAC AGG CAG AGC GAG GTG CCG GCT GGC CAA AGC AGG GGG GCA GCC GGG GTC TCT	674
V V D I E A I K A F L K K C W S V D	204
GTC GTG GAT ATC GAA GCT ATC AAA GCC TTC CTG AAG AAG TGC TGG AGT GTA GAT	728
I S T K E Y A Y L K G A V L F N P D	222
ATC AGT ACG AAG GAG TAT GCG TAC CTG AAA GGA GCT GTG CTG TTC AAC CCA GAT	782
V E G L R C L H Y I Q S L R R E A H	240
GTG GAG GGT TTG CGC TGC CTC CAC TAC ATC CAG TCT CTG CGT CGG GAA GCG CAC	836
Q A L N E H V R L I H R E D T T R F	258
CAG GCT CTG AAC GAG CAC GTC AGG CTG ATC CAC CGC GAG GAC ACG ACG CGG TTC	890
A K L L I A L S M L R A I N P L V V	276
GCC AAA CTG CTC ATA GCT CTG TCC ATG CTG AGG GCC ATC AAC CCG CTG GTG GTC	944
A Q L F F R P V I G A V N I E E V L	294
GCA CAA CTC TTC TTC AGA CCC GTT ATA GGG GCC GTC AAC ATC GAG GAG GTG CTC	998
M E M F Y G K *	312
ATG GAG ATG TTT TAC GGG AAA TAG GTC ACA GTC CAC AGT GCA CTG AGG TGA ATT	1052
CAG CAG ATT CAG ATG CCA GAT GGA CTG AAA TGC TCC AAT CTC TTA TTG AGT ATG	1106
AGA TGT GGG AAG ATG AAG AGG ATA ATA GGG ACT GAA TTT ACA ACT ACT GTG TGT	1160
TGG AGA TCT TCA AGG ACT GTG ACA GTG ACC AAC ACA GCC TAA GTG TGT GCG TGT	1214
GTG TAT GAG AGA ATG AGA AGA GAG AGT GTG TTG AGG GCA AAC TAT GTT ATA ATA	1268
GAG GAA AAA ACA AAG CAG CTG CTG GAA TTC ATA ACT TAT TTG TTT TTA TAT AAA	1322
CTA TTT TTC TAT AAT AAA GAA ATA CTC TCA GAA AAA AAA AAA AAA AAA AAC	1373

Figure 1- Sea bass *DAX1* full length nucleotide sequence and encoding protein. The 5'UTR is 116 bp long and ends with a partially conserved Kozak sequence (GCC ATG GCC), signaling the start codon. The open reading frame (ORF) is 906 bp long. Asterisk

signals the stop codon of the ORF. The 3'UTR is 350 bp long, containing a polyadenylation signal (signaled in bold) and a poly A⁺ tail.

CHAPTER 3

Characterization of two novel Nr0B family members in the European sea bass, *Dicentrarchus labrax*: *DAX2* and *SHP*.

With: Heiner Kuhl, Constantinos C. Mylonas, Deborah M. Power, Richard Reinhardt and Adelino V. M. Canário. Manuscript in preparation.

Abstract

DAX1 and *SHP* are two members of the orphan nuclear receptor family (Nr0B) that have been shown to play determinant roles in mammal gonadal differentiation and function. In fish, these genes have been identified in two species, and have been shown to be expressed during early embryo and larval development. In addition, a putative duplicate *DAX1* gene, named *DAX2*, has been identified in some fish species, although to date, there is still no data on whether it is a functional gene in fish. Thus, the objective of this chapter was to identify *SHP* and *DAX2*, and to determine their mRNA expression during the temperature sex determining period (TSD). *sbSHP* and *sbDAX2* have a conserved gene structure, composed of two coding exons and an intron. *sbSHP* and *SbDAX2* DNA binding domains contain a conserved putative nuclear receptor box (LXXLL-like motif) for physical interaction with other receptors as described earlier in *sbDAX1*. Tissue expression has revealed that *sbDAX2* is only highly expressed in adult testis and pituitary, while the *sbSHP* is mainly expressed in adult ovary and digestive tract tissues. mRNA expression of *sbDAX1*, *sbDAX2*, *sbSHP* between 32 and 60 dph, during TSD, is highly dimorphic between fish grown at either masculinizing (21°C) or feminizing incubating temperature (15°C). Similarly, the mRNA levels of *SF1*, *SOX9.1* and *SOX9.2* were significantly different between fish grown at the two temperatures. At 32 dph there were no differences between temperatures for *sbDAX1*, *sbDAX2*, *SOX9.1*, *SOX9.2* and *SF1* mRNA levels. However, fish grown at 21°C had significantly higher levels of *sbSHP* than those grown at 15°C. Moreover, at 46 dph, *sbSHP*, *sbDAX1*, *sbDAX2*, *SOX9.1*, *SOX9.2* and *SF1* mRNA levels were significantly elevated in fish grown at 21°C. At 60 dph, there were no differences in expression for all genes at the two temperatures. Altogether, the results obtained in this chapter show that *sbDAX1*, *sbDAX2*, *sbSHP*, *SOX9.1* and *SOX9.2* appear to be modulated at early stages by temperature and suggest that they may be part of the gene cascade mediating temperature dependent sex determination in sea bass.

3.1 Introduction

In most mammalian, including humans, mice and marsupials, male gonadal sex is determined genetically by the activation of the *SRY* gene (Sinclair et al., 1990; Koopman et al., 1991). Thus, the expression of *Sry* dictates that the bipotential gonad will develop as testis, while in its absence it will develop as an ovary (Lovell-Badge and Robertson, 1990; Hawkins et al., 1992). In fish, however, an array of sex determining systems has been shown to exist. Many fish also use a chromosomal background for sex determination, e.g. platyfish (Nanda et al., 2000) and lake trout (Reed et al., 1995), however, lack of heterogametic sex chromosomes and a determinant role for environmental factors on sex determination is also a common feature in fish sex determination.

The mechanisms involved in fish genetic sex determination (GSD) and environmental sex determination (ESD) remain largely unknown. So far, several genes have been reported to be involved in mammal GSD (reviewed elsewhere, Swain and Lovell-Badge, 1999). In the past years, homolog to mammalian sex determining genes have been identified in teleost fish, regardless of sex determining system (GSD or ESD), namely the *SOX9*, *DAX1*, *SF1*, *DMRT-1*, among others (Liu et al., 1997; Chiang et al., 2001; Kondo et al., 2002; Wang et al., 2002; Galay-Burgos et al., 2004). However, it is still not clear whether these genes are sex determining as in mammalian, even though the existence of homolog genes in fish suggest that they may have conserved functions. Altogether, the different reports clearly demonstrate that sex determining genes are conserved throughout evolution, and further suggest that, despite the influence of environmental cues on the final outcome of gonadal sex, it is probable that all sex determining systems have at least some genetic component.

One of the potential sex determining genes in fish is the *DAX1* gene (*Nr0B1*). There are several reports implicating the *DAX1* gene in mammal sex determination. Identification of *DAX1* homologs in birds, reptiles and fish and expression of *DAX1* in embryos or larvae during the sex determining period suggests that this gene may also be involved in sex determination of lower vertebrates (Smith et al., 2000; Torres-Maldonado et al., 2002; Wang et al., 2002). Intriguingly, sequences for putative duplicate *DAX1* genes have been found in tilapia (named *DAX2*, genebank accession number ABB88831) and in fugu (Fugu Consortium Scaffold S002663), and are absent from all mammalian genome databases, suggesting that it is a fish specific duplicate gene. However, to date it is yet to be determined if fish express this putative duplicate gene and its function.

In addition, another orphan receptor, member of the *Nr0B* family like *DAX1*, has been isolated in different vertebrates. The small heterodimer partner (*SHP* or *Nr0B2*) is an orphan nuclear receptor that was initially discovered for its role in feedback inhibition of

bile acid synthesis in the liver (Goodwin et al., 2000). However, it has been recently found that this receptor is involved in mammalian reproductive function. Studies in male mice showed that SHP is involved in the control of gonadal germ cell differentiation and androgen production (Volle et al., 2007). In fish, two homolog SHPs have been identified in tilapia and in zebrafish (Wang et al., 2002; Zhao et al., 2006). This nuclear receptor has been found to be expressed in zebrafish embryos (Zhao et al., 2006) and in early larval stages in tilapia (Wang et al., 2002). However, whether it is involved in sex determination in fish is still unknown, but the early expression found in fish together with the described role in gonadal germ cell differentiation in mammalian suggests that SHP is also a potential candidate for a sex determining gene in fish.

Temperature is one of the environmental cues with severe effects on gonadal sex. In fact, studies with reptiles, amphibians and fish have shown that exposure of early embryos, eggs or larvae to different ranges of temperature can bias sex ratios in favor of either sex (reviewed in Baroiller et al., 1999; Pieau et al., 1999; Eggert, 2004). The European sea bass (*Dicentrarchus labrax*), is one of the fish species with labile sex determination, and highly sensitive to temperature influence (Pavlidis et al., 2000; Koumoundouros et al., 2002; Mylonas et al., 2005). Indeed, several studies have shown that the sex ratios within a population can be manipulated by applying different rearing temperature regimes within the range that wild fish experience in natural conditions. However, these studies also show that the same temperature regimes can either bias sex towards female or male development, depending on the developmental stage of the fish and the duration of the temperature treatment. Incubations at low temperatures (13-15°C) during the first 60-93 days post fertilization (dpf) result in female biased populations but the same temperature regime produce male dominant populations when applied either for shorter or longer periods (Pavlidis et al., 2000; Koumoundouros et al., 2002; Mylonas et al., 2005), suggesting that the influence of temperature is related to larval growth and/or development. However, although temperature can bias sex ratios in sea bass populations, no temperature regime is able to produce 100% female or male populations. Different reports have also shown combined effects of temperature and parental influence (Saillant et al., 2002) or with genetic strain (Mylonas et al., 2003; Mylonas et al., 2005) and have also failed to produce 100% female or male populations. Altogether, these studies show that although temperature can influence sex determination in sea bass, it cannot completely override GSD, suggesting that the susceptibility to temperature is, to some extent, dependent on the individual genetic background. Nonetheless, to date, no sex determining gene has yet been identified in this fish species.

The aim of this chapter was to isolate the *SHP* and *DAX2* in the European sea bass and to make a comparative study of the mRNA expression of the 3 members of the

Nr0B family (*DAX1*, *DAX2* and *SHP*) in larvae grown at either feminizing or masculinizing temperatures, during the temperature sex determining period. In addition, we have also studied the expression of 3 other candidate sex determining genes during the temperature sex determining period, *SOX9.1*, *SOX9.2* and *SF1*, in order to clarify the gene cascade that may be involved in sensitive sex determination in sea bass.

3.2 Methods

3.2.1 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 Ramalheite Experimental Station (University of Algarve, Faro, Portugal) prior to sampling in through-flow seawater tanks at 17 ± 2 °C under natural photoperiod. The fish samples used to investigate gene expression during the first 60 dph (days post hatching) came from an experiment already documented in chapter 2. Samples for gene expression analysis were taken at 32, 46 and 60 dph to analyze gene expression. Whole larvae were immediately frozen in liquid nitrogen and stored at -80 °C until used.

3.2.2 RNA isolation and Reverse transcription

Total RNA was isolated from sea bass larvae and different tissues with TRI reagent (Sigma-Aldrich, Madrid, Spain) according to the manufacturer's instructions. Total RNA was treated with the DNA- free Kit (Ambion, California, USA) and 1 µg RNA was reverse-transcribed in 20 µl at 37°C for 60 min., using 40 U of MMLV-RT, 1 mM dNTPs, 6U of RNAGuard RNase inhibitor (Amersham Biosciences) and 1 µg random-hexamers (pd (N) 6, Amersham Biosciences) and subsequently heated at 65°C for 10 min to inactivate the enzyme and stored at -20°C.

3.2.3 Isolation of the sea bass *DAX2* and *SHP* genes and coding region.

High molecular weight DNA was isolated from liver of male sea bass, using a standard extraction method with phenol: chloroform. PCR primers for the *DAX2* amplification (Fw2DAX2/Rv3DAX2) were designed based on the tilapia *DAX2* gene (Genebank Accession number DQ269442) (Table 1).

Table 1 -Primers and RT-PCR conditions for sb*DAX2* and sb*SHP* isolation and gene expression analysis. Primers Fw2DAX2/Rv3DAX2 were used to amplify a partial fragment of *DAX2* from genomic DNA; Primers DAX2 orfFw/Rv and SHP orf Fw/Rv were used to amplify the full sequence of *DAX2* and *SHP* from testis cDNA. All other primer pairs were used for real time PCR analysis in all other experiments.

Primer	Primer sequences (5'-3')	Annealing Temperature
Fw2DAX2 Rv3DAX2/	GGT TGT GGG TCT GGT CCA GGA C AGC CTC CCT GTG AAG TGT CTG G	57° C
DAX2ORFFw DAX2ORFRv	ATG TCG TGT TGT GAG TGC AGA GAC CCG CCC ATA AAA CAT AGC GAG CAC	68° C
SHPORFFw SHPORFRv	ATG GAT AAC GGG TGT CAT TGT TC CTA CCT GCA GAA GAG CAT ATC AAC	64° C
DAX2_RT_Fw DAX2_RT_Rv	ATG AGC ACG TGG GGA CGG TGT A CAG TCC CGC CAC CGC CTC CGT G	60° C
DAX1_RT_Fw DAX1_RT_Rv	GCT CTA AAC GAG CAC GTC AGG C CAC TGG CGG GCT GAT GGC CCT CAG	68° C
SHP_RT_Fw SHP_RT_Rv	TCC TCC TCA CAG CCT CCA TGC TGC GCA GCT CCA GCA GGT TCG CCT GTC	60°C
SF1tiIRT_Fw SF1tiIRT_Rv	GCT GGT CTG AAC TTC TGG TCT TGG GAC AGC TGG ACC TCC TGG CCG	60° C
Sox9.1tiIRT_Fw Sox9.1tiIRT_Rv	CAG CAG GGA CAG CAG CAA CAG CA CAG CAG CTC TGT CTT GAT CTG GGT C	65° C
Sox9.2tiIRT_Fw Sox9.2tiIRT_Rv	GCC TGG ATG TCC AAG CAG C GCT CTG TCT TAA TCT GGG TGG T	62° C
18S_RT_Fw 18S_RT_Rv	CGA TCA GAT ACC GTC GTA GTT TC CCC TTC CGT CAA TTC CTT TA	60° C

High molecular weight genomic DNA was used for PCR amplification of a *DAX2* probe with approximately 340 bp (for PCR conditions see Table 1). The PCR product was sequenced to confirm the identity and subsequently used to screen a Sea bass BAC library (Whitaker et al., 2006). Two positive clones were obtained and mapped to the stickleback, *Gasterosteus aculeatus*, genome and indirectly mapped to a sea bass BACs (Heiner Khul, unpublished), thus The positive BACs were sequenced and the *DAX2* gene was located in contig 51294. A pair of oligo nucleotide primers (DAX2orfFw/Rv, Table 1) was designed flanking the putative coding region of sea bass *DAX2* and used in reverse transcriptase polymerase chain reaction (RT-PCR) to isolate the open reading frame in European sea bass testis cDNA. RT-PCR amplification was carried out in 25 µl reactions containing 1µl of cDNA, 2.5µl of 10x Buffer (Promega), 2.0 mM MgCl₂, 0.04 dNTPs, 0.4 µM sense and anti sense primers, and 0.5 units of taq Polymerase (Promega) (see also Table 1). Products were separated and purified from an agarose gel (1%) with the GFX

PCR DNA and Gel Purification Kit (Amersham Biosciences), cloned in pGemTeasy vector (Promega, Madison, USA) and sequenced.

The European sea bass SHP sequence was obtained by searching the *Dicentrarchus labrax* BAC end sequencing project database (Contig. 91829). A pair of oligo nucleotide primers (SHPorFW/Rv, Table 1) was designed flanking the putative coding region of sea bass SHP identified in the isolated BACs and used in reverse transcriptase-polymerase chain reaction (RT-PCR) to isolate the open reading frame in testis cDNA. RT-PCR reactions were identical to those described for the DAX2 gene (for primer and thermo cycling conditions see table 1). The amplified product was isolated, cloned and sequenced as described for the DAX2.

3.2.4 Multiple sequence alignment and phylogenetic analysis

The accession numbers of the DAX1 sequences used in the alignments and/or in the phylogenetic analysis were from tilapia (AAN17672), medaka (ABQ 09274), sea bass (CAG17628), tetradon (CAG05777), zebrafish (AAY85268), chicken (AF202991), human (NP000466), mouse (CAM21707), rat (NP445769) and the DAX1 from fugu was mapped to Fugu Consortium Scaffold S000386 (<http://fugu.biology.qmul.ac.uk/>). The accession numbers of the DAX2 sequences used in the alignments and phylogenetic analysis were from tilapia (ABB88831), sea bass and the DAX2 from fugu was mapped to Fugu Consortium Scaffold S002663 (<http://fugu.biology.qmul.ac.uk/>). The accession numbers of the SHP sequences used in the alignments and phylogenetic analysis were from tilapia (AAN17673), tetradon (CAG00032), zebrafish (AAH580691 and AAY85285), chicken (AAW33564), human (NP068804), mouse (NP035980), rat (NP476474), medaka (ENSORL00000005570), sea bass and the fugu SHP proteins [designated in this work SHP1(SINFRUT00000151053) and SHP2 (SINFRUT00000151053)]. Alignments were performed using the ClustalW. Phylogenetic analysis was performed using Neighbour joining method with bootstrap from sampling 1000 trees (software: MEGA 3.1, 1993-2005).

3.2.5 Real time PCR

Real-Time PCR analysis was performed using the qPCR MasterMix Plus for SYBR® Green I (Eurogentec) and the Icyler iQ™ Real-Time PCR Detection System (BioRad). Primers for the different analysed genes were designed based on available sequences in different databases (for primer sequences and conditions see table 1).

The PCR reactions used were: 95°C for 10 minutes, followed by 45 successive cycles of 95°C for 10 sec for denaturizing, 61-68 SF1 10 sec. for annealing and 10 sec. for

extension of amplified products. The PCR were performed in 96-well micro plates (Biorad). All measures were performed in duplicates with a variation of less than 5% between replicates.

Standards were prepared by diluting plasmid clones of each of the genes being assessed. A series of 10 fold dilutions were prepared for each standard, starting from 100 pg μl^{-1} miniprep. A regression line was generated by plotting cycle number needed to obtain threshold fluorescence at the mid-logarithmic portion of the amplification against $\log_{10}$ [target gene]. The amount of target present in each reverse transcribed cDNA was quantified by regression analysis, extrapolating each amplified cDNA sample over the standard range. All samples quantified were also analyzed in duplicate. The resulting absolute values were normalized for differences in reverse transcription efficiencies by using the level of 18s rRNA expressed. Negative controls, consisting of RT-PCR mix and without cDNA, were included in all runs to confirm the absence of DNA contamination. Upon reaction completion, data were screened to assess the reproducibility of single measurements.

3.2.6 Statistics

The ratios of all genes to 18S were log transformed and the effects of temperature and age were analysed by two-way Analysis of Variance followed by the Tukey multiple comparison test with a significance level of 5%. Groups were considered significantly different for $p < 0.05$ unless otherwise stated. The results presented in the temperature experiment are expressed as mean $\pm$ standard error of the mean. The results presented in the tissue distribution are expressed as the ratio of gene of interest by the 18S amplicon, and in each tissue, a pool of tissue cDNA obtained from 3 different individuals. In all groups, $n=6$, unless stated otherwise.

3.3 Results

3.3.1 Sea bass *DAX2* and *SHP* gene structure

We have isolated two novel genes in sea bass, with highest similarity to the orphan receptor family *Nr0B*, and named sb*SHP* and sb*DAX2*. The isolated genomic sequence containing the sb*SHP* is 1706 bp long and the genomic sequence containing the sb*DAX2* gene is 1208 bp long. The sb*SHP* and sb*DAX2* genes are composed of a 2 exon/1 intron structure and introns are located at the same position within the ligand binding domain as all genes belonging to the *Nr0B* family described so far (Fig.1).

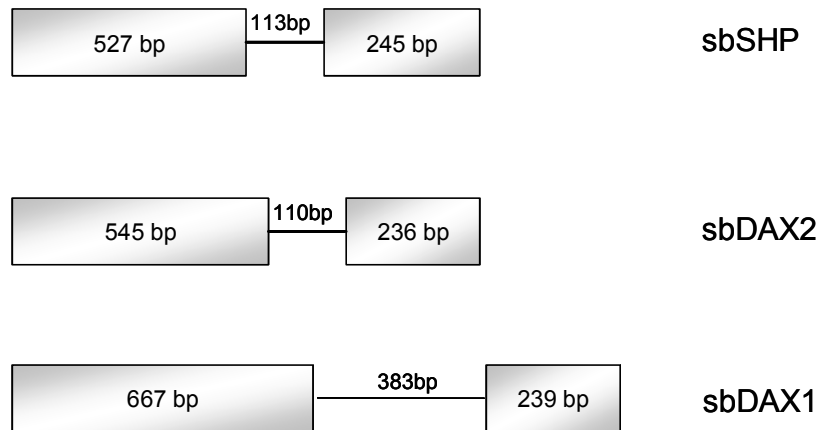


Figure 1– The European sea bass *Nr0B* family members’ gene structure. *SbDAX1* gene structure has been described in chapter 2, sbSHP and sbDAX2 have been isolated from a BAC library, as described in the methods section. Boxes represent exons and lines represent introns. The length of the exons and introns are represented in the figure as number of nucleotides (bp).

3.3.2 Sea bass DAX2 and SHP proteins

The sbSHP the sbDAX2 genes encode putative proteins of 256 and 261 amino acids, respectively (see appendix for details). RT-PCR with specific primers for the sbSHP and sbDAX2 open reading frames have confirmed the size of the open reading frame predicted from the gene structure. Overall, sbDAX2 protein shares highest sequence identity with tilapia, fugu and zebrafish DAX2 proteins (71, 58 and 50% identity, respectively). Curiously, the sbDAX2 shares highest identity to the zebrafish DAX1 (50%) than with the sbDAX1 (43%) (Table 2).

The sbSHP protein shares higher overall sequence identity with other fish SHP than that of sbDAX2 with other DAX2. The sbSHP shares higher identity with the fugu SHP2 and tilapia SHP (84% identity) (Table 3).

Table 2- Comparison of sbDAX2 protein sequence identity with other fish DAX2 and DAX1 proteins. Values are expressed in percentage of identity. The following abbreviations were used In this table: Sbass (sea bass) and D rerio (zebrafish).

	DAX1 Tilapia	DAX1 Medaka	DAX1 Sbass	DAX1 Fugu	DAX1 Tetradon	DAX1 D rerio	Dax2 Sbass	DAX2 Tilapia	DAX2 Fugu
DAX1 Tilapia	ID	83	84	80	78	57	43	41	36
DAX1 Medaka	83	ID	78	76	75	56	44	41	36
DAX1 Sbass	84	78	ID	84	81	56	43	40	34
DAX1 Fugu	80	76	84	ID	89	54	40	38	34
DAX1 Tetradon	78	75	81	89	ID	54	41	38	34
DAX1 D rerio	57	56	56	54	54	ID	50	47	38
Dax2 Sbass	<u>43</u>	<u>44</u>	<u>43</u>	<u>40</u>	<u>41</u>	<u>50</u>	<u>ID</u>	<u>71</u>	<u>58</u>
DAX2 Tilapia	41	41	40	38	38	47	71	ID	54
DAX2 Fugu	36	36	34	34	34	38	58	54	ID

Table 3- Comparison of sbSHP protein sequence identity with other fish SHP proteins. Values are expressed in percentages. The following abbreviations were used In this table: Sbass (sea bass) and D rerio (zebrafish).

	SHP Tilapia	SHP Medaka	SHP Sbass	SHP2 Fugu	SHP Tetradon	SHPA D rerio	SHP B D rerio	SHP1 Fugu
SHP Tilapia	ID	77	84	81	79	61	36	43
SHP Medaka	77	ID	76	74	72	58	35	40
SHP Sbass	<u>84</u>	<u>76</u>	<u>ID</u>	<u>84</u>	<u>81</u>	<u>60</u>	<u>37</u>	<u>41</u>
SHP2 Fugu	81	74	84	ID	88	60	37	41
SHP Tetradon	78	72	81	88	ID	60	35	41
SHPA D rerio	61	58	60	60	60	ID	37	37
SHP B D rerio	36	35	37	37	35	37	ID	31
SHP1 Fugu	43	40	41	41	41	37	31	ID

3.3.3 Multiple sequence alignment and phylogenetic analysis

Multiple sequence alignment shows that sbSHP and sbDAX2 proteins have the Nr0B family members' atypical DNA binding domain (DBD) without zinc fingers, but they maintain a conserved nuclear receptor box (LXXLL motifs). Thus, the sbDAX2 has a well conserved classical LXXLL motif (SILYSLL), while the sbSHP has an LXXLL-like motif (PILFKIL) (Fig.2). Moreover, the DBD of all fish DAX2 and SHP proteins are smaller than the DAX1 proteins as they lack the polyglutamine stretch identified in the sbDAX1. Both sbSHP and sbDAX2 proteins contain conserved ligand binding domain (LBD) and AF-2 core, as sbDAX1.

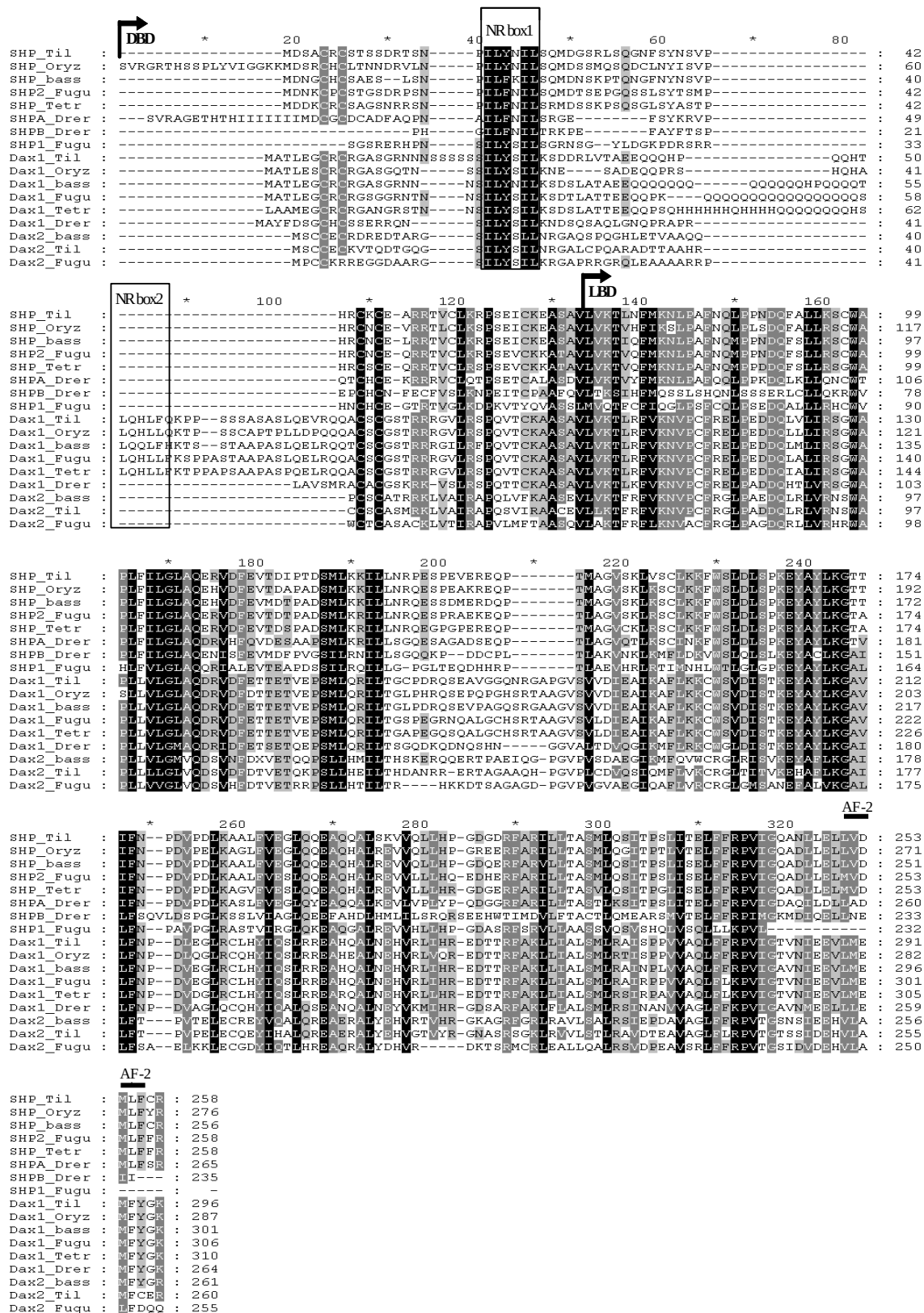


Figure 2 – Multiple sequence alignment of fish Nr0B family members DAX1, DAX2 and SHP protein in fish. Identical and similar amino acids are marked with asterisks and dots, respectively. The DNA binding domain (DBD), ligand binding domain (LBD), AF-2 core and nuclear localization signals (NR box 1 and 2) are also indicated in the alignment.

Phylogenetic analysis shows that sbDAX2 cluster together with other fish DAX2 proteins and separate from the fish DAX1 (Fig. 3). The sbSHP protein clusters with the SHP proteins from other fish species, but interestingly, there are duplicate genes encoding the SHP in fugu and zebrafish, and the sbSHP protein, as all other fish, are more similar to the SHPA from zebrafish and fugu, than to the duplicate forms SHPB in zebrafish and fugu.

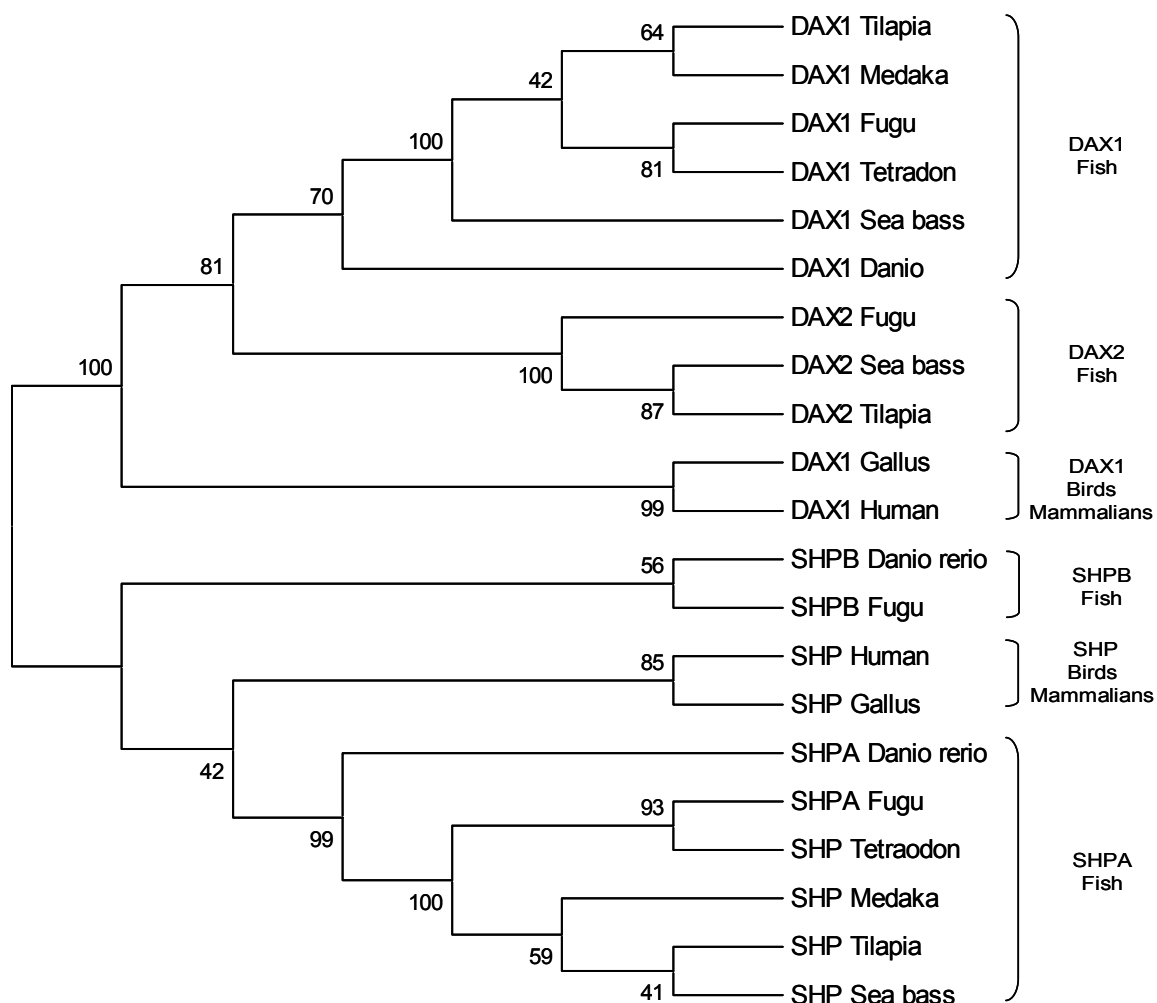


Figure 3 –Phylogenetic tree of sbDAX2 and sbSHP proteins with vertebrate’s Nr0B family members. Consensus tree obtained by Neighbour joining analysis with corresponding bootstrap values from sampling 1000 trees. Sequence’s accession numbers are documented in the Methods section.

3.3.4 Tissue distribution of *sbDAX2*, *sbSHP* and *sbSF1* mRNA expression

The pattern of *sbDAX2* expression in different tissues and gonads from females and males was determined by real time PCR (Fig. 4). The *sbDAX2* mRNA was highly expressed in male gonads, and only residual levels could be detected in female gonads. Similarly, mRNA expression of *DAX2* was also detected in pituitary of male and female fish, although, males presented higher levels than females, as seen in gonadal tissues. In all other tissues analyzed, the *DAX2* mRNA levels were below or reaching the threshold detection levels of the assay.

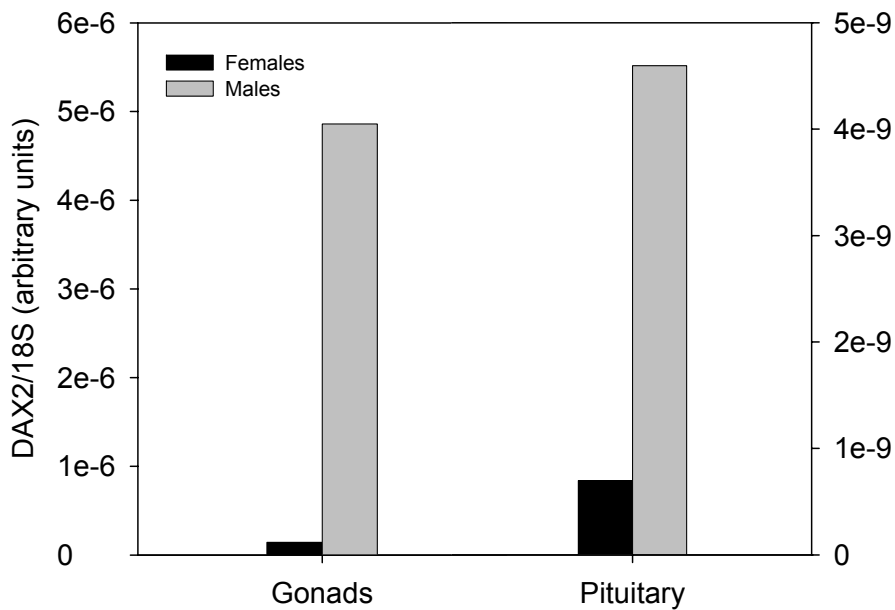


Figure 4 – *DAX2* mRNA expression in female and male adult gonads and pituitary. Data are expressed as the ratio of real time PCR amplification of *sbDAX2* relative to the 18S amplification. Each tissue consists of a pool of 3 individual ovaries or testis and of a pool of 3 individual female or male pituitaries.

A dimorphic pattern of expression was also found in *sbSHP* mRNA levels in the gonads, as females expressed high levels of *SHP* mRNA, while in males no expression could be found (Fig. 5). We have also detected very low levels of *sbSHP* mRNA in liver, esophagus, stomach, duodenum, mid intestine and rectum.

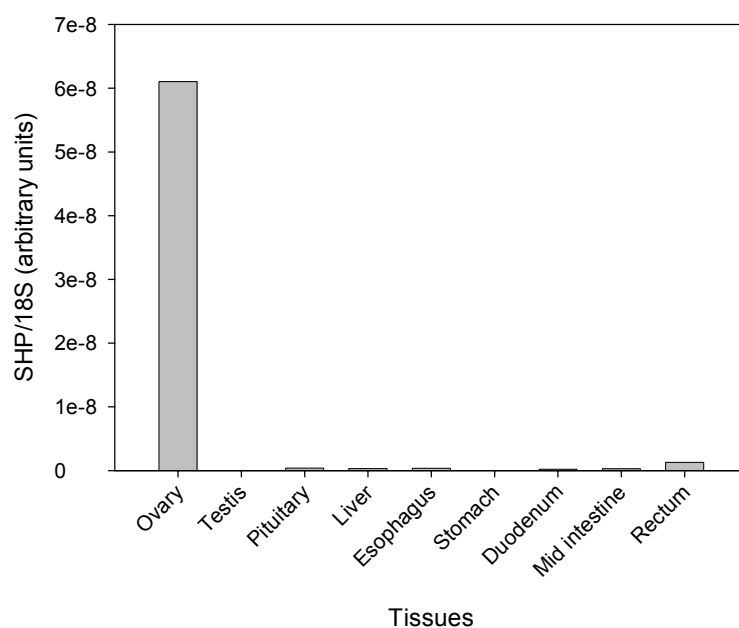


Figure 5– *SHP* mRNA expression in different tissues of adult sea bass. Each tissue consists of a pool of mRNA from both female and male tissues, with the exception of ovary and testis.

The mRNA levels of *SF1* present a dimorphic expression in the gonads of adult sea bass, and testes express higher levels than ovaries (Fig. 6).

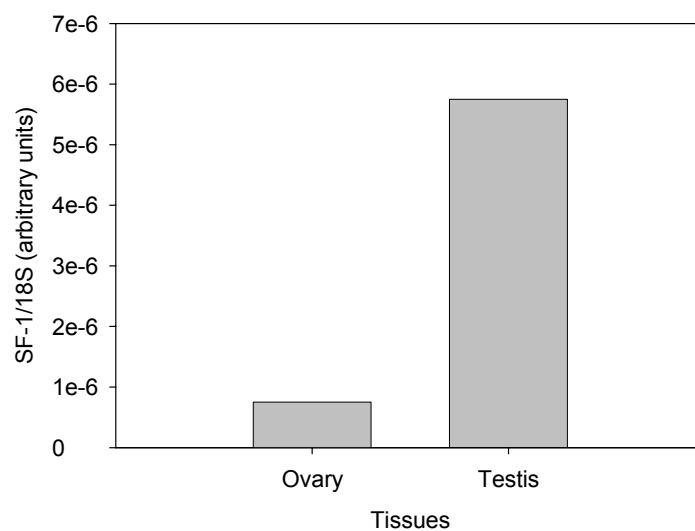


Figure 6- *SF1* mRNA expression in male and female gonads of adult sea bass. Each tissue consists of a pool of mRNA from either 3 ovaries or 3 testis.

3.3.5 *DAX2* and *SHP* mRNA expression during early development

The *sbDAX2* mRNA levels could be detected as early as 32 dph and until the end of the labile period for temperature sex determination (TSD) in sea bass (60dph) (Fig. 7). Fish grown at 15°C presented low *DAX2* mRNA levels from 32 to 46 dph, however, at 60 dph, *sbDAX2* is significantly increased when compared to all other developmental stages ($p<0.01$). Fish grown at 21°C presented an increase in mRNA expression from 32dph to 46 dph, although no significant differences could be found. However, at 46 dph, *sbDAX2* mRNA level from fish grown at 15°C are significantly lower than those detected in fish grown at 21°C ($p<0.05$). Nonetheless, the *DAX2* mRNA expression profile was significantly affected by the temperature regime applied in culture conditions ($p<0.02$).

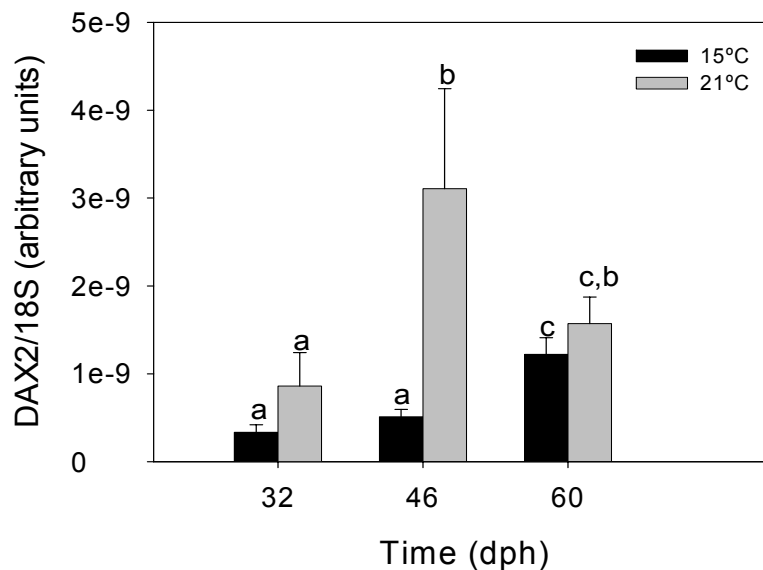


Figure 7- *DAX2* mRNA expression during early development in European sea bass.

Real time PCR of *DAX2* mRNA levels, relative to 18S in whole sea bass larvae ($n=6$ at each sampling point). Larvae were grown at either 15°C or 21°C resulting, respectively, in 77.5% and 28% females. Significant differences between temperatures at each developmental stage are marked with different letters when $p<0.05$.

The *sbSHP* mRNA levels could be detected as early as 32 dph and until the end of TSD period in sea bass (60dph) (Fig. 8). Fish grown at 15°C presented low *SHP* mRNA

levels from 32 to 60 dph, and no significant differences could be found at any developmental stage analyzed. Fish grown at 21°C presented an increase in mRNA expression from 32dph to 46 dph, although no significant differences could be found. At 60dph, however, *SHP* mRNA levels were significantly decreased when compared to those detected at 46 dph ($p<0.05$). Nonetheless, the *SHP* mRNA expression was significantly affected by the temperature regime applied in culture conditions ($p< 0.05$).

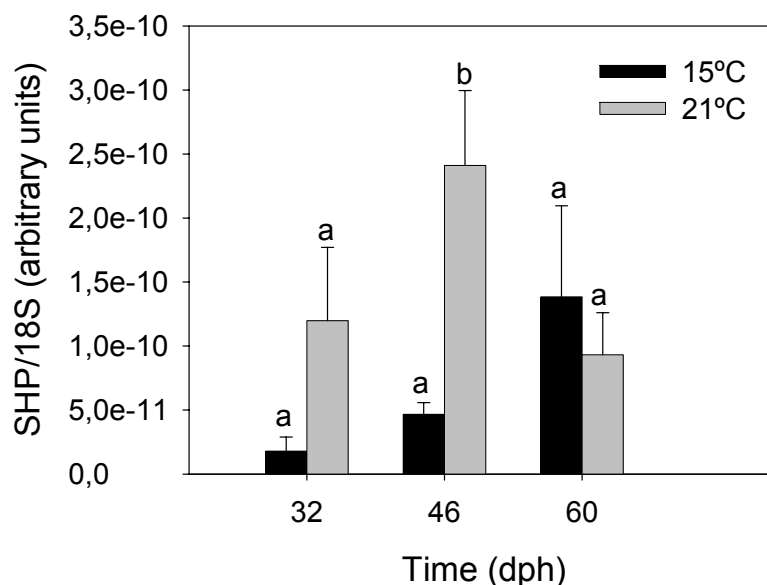


Figure 8- *SHP* mRNA expression during early development in European sea bass. Real time PCR of *SHP* mRNA levels, relative to 18S in whole sea bass larvae ($n=6$ at each sampling point). Larvae were grown at either 15°C or 21°C resulting, respectively, in 77.5% and 28% females. Significant differences between temperatures at each developmental stage are marked with different letters when $p<0.05$.

3.3.6 *DAX1*, *SF1*, and *SOX9* genes mRNA expression during early development

The *SF1* mRNA levels could be detected as early as 32 dph and until the end of the labile period for TSD in sea bass (60dph) (Fig. 9). Fish grown at 15°C presented similar *SF1* mRNA levels from 32 to 60 dph, although they were slightly decreased at 46dph. Fish grown at 20°C, also expressed *SF1* mRNA throughout this period, however, at 60dph, the levels were significantly decreased when compared to 46dph ($p<0.01$). Also, at 46dph, fish grown at 21°C have significantly higher *SF1* mRNA levels than fish grown at lower temperatures ($p<0.001$). Nonetheless, the *SF1* mRNA expression profile was not significantly affected by the temperature regime applied in culture conditions.

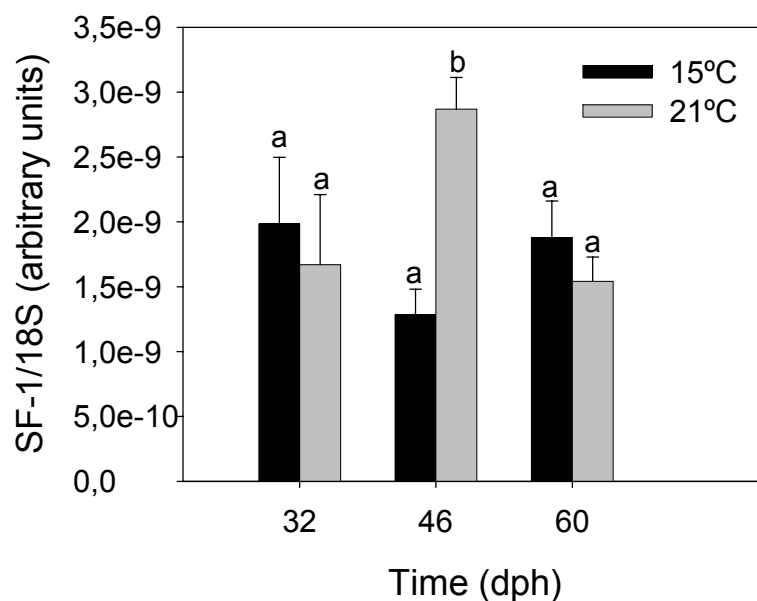


Figure 9- *SF1* mRNA expression during early development in European sea bass. Real time PCR of *SF1* mRNA levels, relative to 18S in whole sea bass larvae ($n=6$ at each sampling point). Larvae were grown at either 15°C or 21°C resulting, respectively, in 77.5% and 28% females. Significant differences between temperatures at each developmental stage are marked with different letters when $p<0.05$.

The *sbDAX1* mRNA levels could be detected as early as 32 dph and until the end of the labile period for TSD in sea bass (60dph) (Fig. 10). Fish grown at 15 °C presented low *DAX1* mRNA levels from 32 to 60 dph. Fish grown at 21°C presented significantly higher mRNA levels at 46 dph than at any other developmental stage analyzed ($p<0.01$). At 46 dph, the *sbDAX1* mRNA levels of fish grown at 21°C were significantly higher than those found in fish grown at lower temperature ($p<0.001$). Thus, the *DAX1* mRNA expression profile was significantly affected by the temperature regime applied in culture conditions ($p<0.001$).

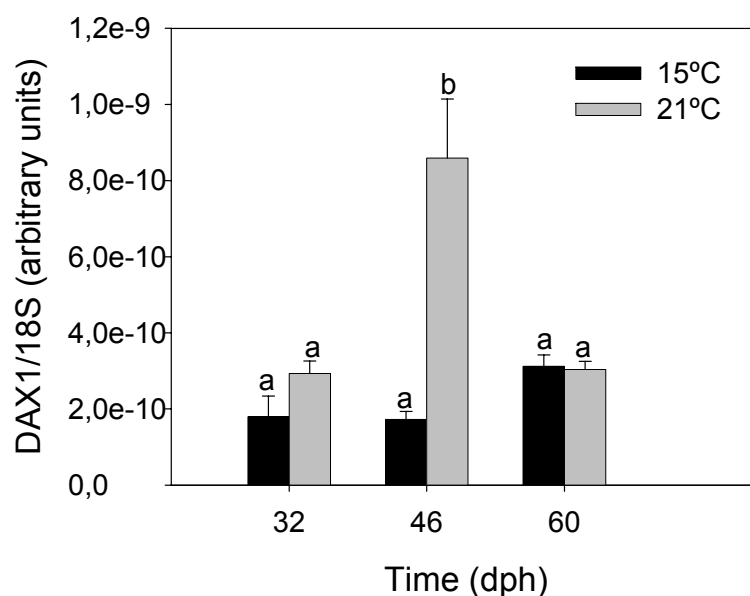


Figure 10- *DAX1* mRNA expression during early development in European sea bass.

Real time PCR of *DAX1* mRNA levels, relative to 18S in whole sea bass larvae ($n=6$ at each sampling point). Larvae were grown at either 15°C or 21°C resulting, respectively, in 77.5% and 28% females. Significant differences between temperatures at each developmental stage are marked with different letters when $p<0.05$.

The *sbSOX9.1* mRNA levels could be detected as early as 32 dph (Fig. 11). Fish grown at 15 °C presented low *SOX9.1* mRNA levels at 32 dph. These levels were significantly increased at 46 dph ($p<0.001$), but are maintained constant thereafter. Fish grown at 21°C presented a significant increase in mRNA expression from 32dph to 46 dph ($p<0.01$) but are subsequently decreased until 60dph. At 46 dph, the *sbSOX9.1* mRNA levels of fish grown at 21°C were significantly higher than those found in fish grown at lower temperature ($p<0.01$). Thus, the *SOX9.1* mRNA expression profile was significantly affected by the temperature regime applied in culture conditions ($p<0.02$).

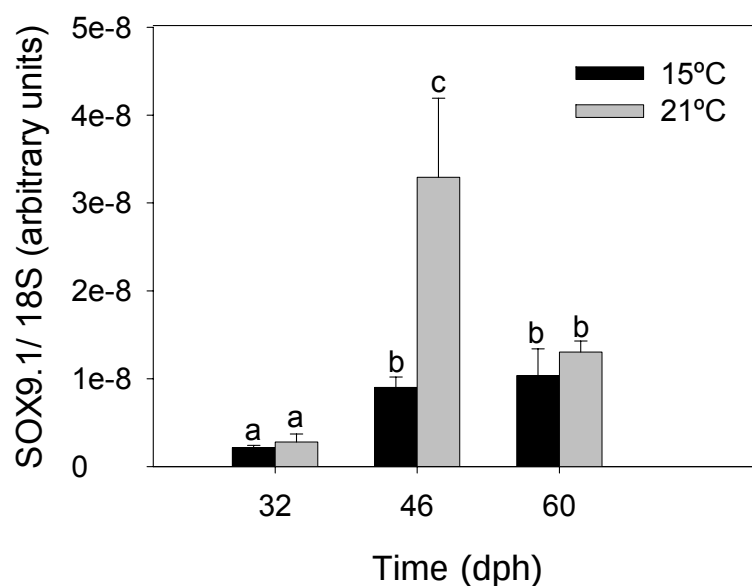


Figure 11- SOX9.1 mRNA expression during early development in European sea bass. Real time PCR of SOX9.1 mRNA levels, relative to 18S in whole sea bass larvae ($n=6$ at each sampling point). Larvae were grown at either 15°C or 21°C resulting, respectively, in 77.5% and 28% females. Significant differences between temperatures at each developmental stage are marked with different letters when $p<0.05$.

Fish grown at 15°C presented low sbSOX9.2 mRNA levels from 32 until 60 dph (Fig. 12). Fish grown at 21°C presented a significant increase in mRNA expression from 32dph to 46 dph ($p<0.01$) but subsequently decreased. At 46 dph, the sbSOX9.2 mRNA levels of fish grown at 21°C were significantly higher than those found in fish grown at lower temperature ($p<0.01$). Thus, the SOX9.2 mRNA expression profile was significantly affected by the temperature regime applied in culture conditions ($p<0.02$).

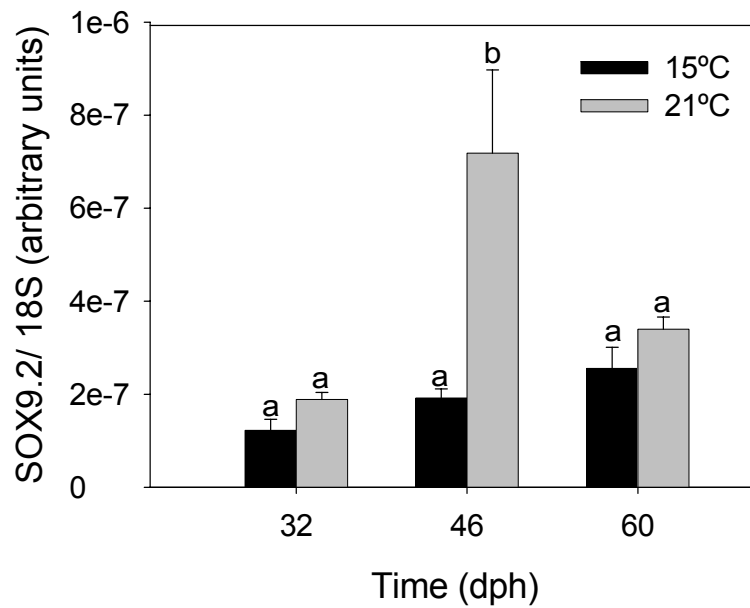


Figure 12- SOX9.2 mRNA expression during early development in European sea bass. Real time PCR of SOX9.2 levels, relative to 18S in whole sea bass larvae ($n=6$ at each sampling point). Larvae were grown at either 15°C or 21°C resulting, respectively, in 77.5% and 28% females. Significant differences between temperatures at each developmental stage are marked with different letters when $p<0.001$.

3.4 Discussion

In this chapter, we have isolated two novel members of the *Nr0B* family in the European sea bass, and characterized the gene structure, tissue distribution and ontogenic expression of *sbDAX2* and *sbSHP*. The *sbDAX2* and *sbSHP* genes have a well conserved two exon structure, separated by a single intron and conserved splice sites located at the same region of the ligand binding domain of *sbDAX1* and as all other *DAX1* and *SHP* genes described in different species. The *sbDAX2* gene encodes a 261 amino acids protein that shares highest overall sequence identity and similarity to other *DAX2* homolog genes in tilapia and fugu. Phylogenetic analysis shows that *sbDAX1* and *sbDAX2* cluster with other fish homolog *DAX1* and *DAX2*, respectively, and separate from the *SHP* genes. These results suggest that *DAX1* and *SHP* have arisen from a common ancestral gene, and further demonstrates that *DAX2* genes have probably arisen from fish specific gene duplication. Interestingly, the *DAX1* genes from human and chicken lay outside of the fish *DAX1* and *DAX2* gene clusters, suggesting that this gene duplication in fish is a recent event. The *sbSHP* gene encodes a 256 amino acid protein that shared

highest overall identity to other the SHP described in tilapia (Wang et al., 2002). However, SHP has also been duplicated in zebrafish and in fugu genomes. In these species there are two SHPs, named SHPA and SHPB. SHP from birds and mammalian cluster with sea bass, tilapia, medaka, tetradon SHPs and with fugu and zebrafish SHPA. Altogether, these data suggest that the Nr0B family has been subjected to two genome duplication events. Nevertheless, further work is still necessary to determine whether there is also a duplicate gene for the SHP in sea bass.

Similar to what was documented in chapter 2 for sbDAX1, the sbDAX2 and sbSHP genes lack the nuclear receptor's characteristic zinc-finger motif in the DNA-binding domain (DBD). However, in the DBD, they maintain one of the 3 NR boxes containing LXXLL or LXXLL-like motifs that are characteristic of the DBD of the mammal DAX1. The sbDAX2 is the only gene in fish *Nr0B* members that possesses a classical LXXLL motif (SYLYSLL) as in the human DAX1. In fact, the sbSHP and all other fish DAX1, DAX2 and SHP members contain a LXXLL-like motif, as seen in amphibians (Sugita et al., 2001), reptiles (Western et al., 2000), and birds (Smith et al., 2000). Interestingly, the sbDAX1 NR box that is conserved in its DBD had been shown to align with the NR box 2 of the mammal *DAX1* genes (chapter 2). Similarly, the NR box conserved in both sbDAX2 and sbSHP proteins also align with the same NR box (data not shown). However, when we aligned fish *Nr0B* members without the mammalian genes, we found that all fish DAX1 genes, except for the zebrafish DAX1, have a long stretch of amino acid sequence that is lacking from all fish DAX2 and SHP genes. Moreover, within this stretch, all fish *DAX1* genes appear to possess a second putative NR box with LXXLL or LXXLL-like motifs and a third putative NR box with a LXXLL-like motif in fugu and sea bass DAX1. In mammalian DAX1, both the classical LXXLL motif and the LXXLL-like motifs have been shown to be able to interact with other nuclear receptors, although with different binding affinities (Zhang et al., 2000). Whether the fish DAX1, DAX2 and SHP proteins also use these NR boxes to interact with other receptors is still to be determined, however, conservation of 3 putative LXXLL-like motifs in fish DAX1 and not in DAX2, together with the restricted mRNA distribution suggests that the latter has evolved separately and probably has acquired different functions in fish.

sbSHP and sbDAX2 proteins contain a highly conserved ligand binding domain (LBD) and AF-2 core, as sbDAX1, which suggests that these three receptors probably interact with other proteins in a similar way. Although further studies are still necessary to determine the function of these receptors in sea bass, the presence of NR boxes in their DBD and the well conserved LBD and AF-2 cores suggests that these receptors probably interact with other proteins using these conserved regions as described for mammalian DAX1 and SHP proteins (Lalli et al., 1997; Crawford et al., 1998; Johansson et al., 1999).

Interestingly, *sbDAX2* mRNA expression appears to be very specific in fish. In fact, although we have detected *DAX2* mRNA expression in the same tissues as *DAX1*, the mRNA levels were detected at the limit of detection of the RT-PCR, except in the gonads and pituitary. In these tissues *DAX2* mRNA levels are highly dimorphic, as testis and pituitary in males express higher levels than ovary and pituitary in females. This expression pattern is the opposite to that of *sbDAX1* (see chapter 2) and tilapia (Wang et al., 2002) where female pituitary and ovary express higher levels of *DAX1*. The *DAX1* gene appears to have quite ambiguous functions in mammalian gonadal development as over expression of this gene leads to male sex reversal (Swain et al., 1998) but, *DAX1* mutations (Muscatelli et al., 1994) or gene knockout results in abnormal testicular morphology and sterility (Jeffs et al., 2001a; Jeffs et al., 2001b; Bouma et al., 2005). Considering the dimorphic expression pattern of *sbDAX1* and *sbDAX2*, it is likely that these receptors have evolved independently and have probably acquired female and male specific functions in fish. Thus, unlike mammalian, where a single *DAX* gene is both involved in female and male sexual differentiation, and is able to regulate independently female and male sex determining genes (see Clipsham and McCabe, 2003 for review), the sea bass possesses two separate *DAX* genes, with dimorphic expression in the gonads and possibly with sex specific functions.

The mammalian *DAX1* regulatory actions in the gonads are correlated to the presence and activity of the nuclear receptor steroidogenic factor 1 (*SF1*). Mammalian *DAX1* and *SF1* have been shown to be expressed in the early bipotential gonad and throughout sexual differentiation (see Val and Swain, 2005 for review), and also to regulate several common target genes within the steroidogenic pathway (Lalli et al., 1997a; Lalli et al., 1998). We have analyzed the *SF1* mRNA levels in adult male and female gonads, and found that, similar to the *sbDAX2*, the *SF1* mRNA levels are more expressed in males than in females. Thus, these data suggests that *sbDAX2* and *SF1* may act together to promote male reproductive function while *sbDAX1* may be more involved in female reproductive function.

In sea bass, sex determination is modified by exogenous sex steroid during the labile sex differentiation period (between 57 and 137 post-fertilization), suggesting that the phenotypic outcome of the gonads requires the production of androgens or estrogens (Piferrer, 2001; Devlin and Nagahama, 2002). During this period, steroidogenic enzymes and steroid receptors are expressed in the developing gonad. During this period, maculinization is accompanied by increased expression of CYP11B1 and AR (Blazquez and Piferrer, 2005; Socorro et al., 2007) and feminization by increased activity of steroid enzyme aromatase (Blazquez and Piferrer, 2005) and transcription of ERs (Halm et al., 2004). At this stage the male and female brain and pituitary axis are fully developed and

present dimorphic levels of *sbGnRH* and *sGnRH*, reflecting the sexual differentiation of the brain (Moles et al., 2007). In concert, these studies show that the activation of both steroid production and of the brain-pituitary axis is necessary or accompanies sexual differentiation. However, sex ratios are also influenced by temperature and when different temperature regimes are applied prior to the sex differentiation period (prior to 90 dph) (Pavlidis et al., 2000; Koumoundouros et al., 2002; Mylonas et al., 2005). These studies have shown that female dominant populations are obtained using low temperature regimes and conversely, high temperature yield male dominant populations. Despite the reported effects on sex ratios, none of the studies has been able to obtain only female populations, suggesting that some individuals have a genetic background that is not overridden by temperature. Thus, the influence of temperature on gene expression can help to clarify which genes are involved in the early sex determining period.

Earlier studies in sea bass larvae had reported that *CYP11B1* and *aromatase* are already expressed at 4 dph, suggesting that androgen and estrogen production in extra gonadal tissues from an early age (Socorro et al., 2007) at a time when *sbDAX1* is already expressed (chapter 2). However, none of these studies have shown any influence of temperature on mRNA levels, probably due to low sensitivity of the quantification methods used (semi quantitative PCR). In the present chapter, we have analyzed the mRNA expression of *sbDAX2*, *sbSHP*, *SF1*, *sbSOX9.1* and *sbSOX9.2* during the temperature sensitive sex determining period (32-60 dph) and reanalyzed the *sbDAX1* expression levels using the more sensitive real time PCR. At female producing temperatures (15°C), *sbDAX1*, *sbDAX2*, *sbSHP* and *SOX9.2* mRNA levels are maintained low from 32 dph until 60 dph. However, the *SOX9.1* mRNA levels increase significantly from 32 to 46 dph, and are maintained unaltered until 60 dph. The *SF1* mRNA levels are not significantly altered throughout all developmental stages. Conversely, all genes displayed a significant increase in mRNA expression at 46 dph at masculinizing temperatures (21°C), followed by a decrease until 60 dph. Thus, the expression profile of these genes at masculinizing temperatures is significantly different from that of fish grown at feminizing temperatures.

Interestingly, in sea bass, the temperature regimes that are applied until 60-90 dph are those that are more effective in creating female dominant or male dominant populations (Mylonas et al., 2005). In addition, the results presented in this chapter suggest that the effect of temperature on sea bass sex ratios can be directly or indirectly related to its influence on gene transcription at early stages of development. Thus, considering all the data obtained in this chapter, we suggest that *DAX1*, *DAX2*, *sbSHP*, *SOX9.1* and *SOX9.2* may be involved within the sex determining gene cascade acting during the TSD in sea bass. Nonetheless, it is noteworthy the fact that despite the

delayed gene expression and also the feminizing effect of low temperatures, the sex ratios obtained show that the effect of temperature cannot produce 100 % female populations. Thus, these results suggests that either temperature cannot override the genetic background in a significant amount of individuals, or that the effect of temperature by itself is not the sole responsible for sex biased populations in sea bass.

Recently, it has been shown that exposure to lower, feminizing, temperatures during the TSD period results in significant decreases in sea bass growth (Blazquez et al., 1999; Saillant et al., 2002; Mylonas et al., 2005). Furthermore, size has also been shown to be a critical marker for sexual differentiation since larger fish develop as females (Blazquez et al., 1999). These studies suggest that the reduction of growth at low temperatures could be directly or indirectly affecting sexual differentiation. In mammalian, testes formation relies upon the activation of *SRY* (Koopman et al., 1991), while female pathway proceeds in the absence of male-promoting signals. However, it has been recently proposed that female sex determination is only determined when the germ cells enter meiosis (Yao et al., 2003). Thus, in the bipotential gonad, the entry of the germ cells into meiosis would initiate the ovarian pathway and block testis development, while, *SRY* opposes this pathway by initiating cord formation prior to meiosis, thereby sequestering germ cell inside testis cords and blocking meiosis. Similarly, in an inbred strain of Medaka (Hd-rR), the first signs of sex difference in germ cells is identified at stage 38 (Kobayashi et al., 2004) after which, germ cells enter mitotic arrest in XY fry, while they proceed to meiosis after hatching in XX fry (Kobayashi et al., 2004; Suzuki et al., 2005), suggesting that this is a conserved process amongst vertebrates. Interestingly, (Nef et al.) (2005) found the cyclin-dependent kinase inhibitor *Cdkn1a* (*p21*) and *Cdkn1b* (*p27*), which regulate cell cycle, to be over expressed in XX as compared to XY gonads before ovarian development. Moreover, (Schmahl and Capel) (2003) have recently shown that inhibition of germ cell proliferation in XY gonads within an 8-h period of gonadal development (E11.2) leads to ovarian differentiation, suggesting that germ cell proliferation within a developmental window is essential to initiate the male pathway. Thus, it is possible that in sea bass, the reduction of growth seen at low temperatures could be suppressing germ cell proliferation and consequently, preventing male differentiation. This hypothesis could also explain why, at low temperatures, gene expression is kept at low levels, while at high temperature gene expression appears to be activated and increasing between 32 to 46 dph, which is the period at which the first germ cells proliferate to form the primordial gonad. Moreover, the decrease in *SF1* mRNA expression at feminizing temperature in this developmental timing is also compatible with this hypothesis since the *SF1* has been shown to be involved in germ cell migration to the urogenital ridge and formation of the indifferent gonad (Hanley et al., 1999). Altogether, our data suggest that low temperature

regimes suppresses early gene expression in sea bass larvae, when germ cells start to appear (Roblin and Bruslé, 1983), and thus male development is suppressed in favor of female pathway.

To date, only one *SHP* has been isolated and characterized in a fish species. In tilapia, the *SHP* is expressed in most of the tissues analyzed, although it presented higher expression in the liver and intestine (Wang et al., 2002). Similarly, the *sbSHP* mRNA expression was also detected in different sea bass tissues although the tissue with highest expression was the ovary, followed by rectum, liver and other tissues of the digestive tract. In tilapia, RT-PCR analysis has also shown that *SHP* is more expressed in ovary than in the testis (Wang et al., 2002). Interestingly, a large scale gene expression analysis in mouse has shown that the *SHP* mRNA is significantly higher in tissues from the digestive tract, thyroid and ovary (Bookout et al., 2006). Altogether, the *SHP* expression profile appears to be maintained between fish and mammalian, suggesting that both *sbSHP* and tilapia *SHP* have similar functions as mammalian homolog genes.

Recently, (Volle et al.) (2007) have studied the role of *SHP* in mice sexual differentiation and have shown that *SHP* is determinant for germ cell differentiation. In that study *SHP* was able to inhibit the retinoic acid (RA) signaling pathway and the expression of known markers of germ cell differentiation in the early gonad of XY males. Furthermore, they have shown that in the absence of *SHP* expression, RA signaling leads the germ cells into the precocious entry on the meiotic pathway, which accelerates gonadal development and maturation due to earlier increase in steroid enzymes and testosterone production. Additionally, *in vitro* studies have also shown that *SHP* is able to repress the BETA2-dependent activity of the *cdk inhibitor p21* gene (Kim et al., 2004), which has been shown to be over expressed only in germ cells that are differentiating into ovaries (Nef et al., 2005). In concert, these studies suggest that *SHP* may be a determinant in the regulation of the cell cycle in germ cells and thus regulate testicular development in mammalian. Interestingly, *SHP* has also been shown to be involved in thermogenesis control in brown adipocytes (BAT) in mammalian (Wang et al., 2005). Exposure to low temperatures inhibited the *SHP* mRNA transcription in the liver, consistent with its negative regulatory role in BAT (Wang et al., 2005). Thus, in mammalian, *SHP* mRNA levels are directly influenced by temperature. This temperature influence is also shown in sea bass larvae. In fact, *SHP* mRNA levels are significantly lower at 15°C (masculinizing temperature) than at 21°C (feminizing temperature), during early development (32- 46dph), suggesting that in fish it is also target for temperature regulation. This expression pattern, together with the demonstrated role in germ cell differentiation in mice male gonads, further suggests that the effect of temperature on gonadal sex differentiation is probably due to its influence on growth and germ cell differentiation.

Thus, one possible explanation for the observed effect of temperature on sea bass sex determination is that low temperatures suppress germ cell proliferation in the urogenital ridge, possibly through inhibition of transcription of genes that regulate cell cycle, namely *SHP*. The decreased *SHP* mRNA levels could lead to germ cell mitotic arrest, which would, by default, proceed to germ cell meiotic differentiation and thus favor female differentiation. The delayed germ cell proliferation rate could explain why *DAX1*, *DAX2*, *SOX9.1*, *SOX9.2* and *SF1* transcription is suppressed at low temperatures. Moreover, a direct influence of temperature on germ cell differentiation and proliferation could explain why low temperatures are more effective in favoring the female differentiation prior to 90 dph. Indeed, after 90 dph, the bipotential gonad is already committed to becoming either female or male, which is corroborated by the up regulation of estrogen receptors (*ERs*) (Halm et al., 2004), *CYP11B1* and androgen receptors (*ARs*) (Blazquez and Piferrer, 2005; Socorro et al., 2007) mRNA expression, as well as with the increased sensitivity to either exogenous estrogen or androgen. Thus, any influence of temperature would be possibly reflected in altered steroid production and/or steroid receptor transcription, and would most likely affect the maturation of the gonad but would not lead to sex reversal.

In summary, we have isolated two new members of the *Nr0B* family in sea bass, named *sbDAX2* and *sbSHP*. These genes have conserved gene structure and are highly similar to other *DAX2* and *SHP* described in fish. In adult fish, *sbDAX2* is highly expressed in testis and pituitary, while *sbSHP* is mainly expressed in ovary and digestive tract. mRNA expression of duplicate *DAX* genes, *sbDAX1* and *sbDAX2*, *sbSHP*, as well as other candidate sex determining genes, *SF1*, *sbSOX9.1* and *sbSOX9.2*, during the sex determining period is strongly influenced by temperature. At masculinizing temperatures, all genes analyzed are up regulated between 32 and 46 dph, while at feminizing temperatures all these genes are apparently suppressed. Altogether, these data suggest the *sbDAX1*, *sbDAX2*, *sbSHP*, *SOX9.1* and *SOX9.2* are likely involved in early sex determination gene cascade in sea bass, and also that, the effectiveness of using different temperature regimes during early larvae development to skew sex ratios is, to some extent, explained by its capability for directly or indirectly altering the expression of sex determining genes (*DAX1*, *DAX2*, *SHP*, *SOX9.1* and *SOX9.2*).

APPENDIX

sbDAX2

M	S	C	C	E	C	R	D	R	E	D	T	A	R	G	15
ATG	TCG	TGT	TGT	GAG	TGC	AGA	GAC	CGG	GAG	GAC	ACG	GCG	CGG	GGG	45
S	I	L	Y	S	L	L	N	R	G	A	Q	S	P	Q	30
AGC	ATC	CTC	TAC	AGC	CTC	CTC	AAC	AGA	GGC	GCT	CAG	AGC	CCG	CAG	90
G	H	L	E	T	V	A	A	Q	Q	P	C	S	C	A	45
GGA	CAC	CTG	GAG	ACC	GTC	GCC	GCG	CAA	CAG	CCC	TGC	TCC	TGC	GCG	135
T	R	R	K	L	V	A	I	R	A	P	Q	L	V	F	60
ACC	AGG	AGG	AAA	CTG	GTC	GCC	ATT	CGG	GCT	CCG	CAA	TTA	GTT	TTC	180
K	A	A	S	E	V	L	V	K	T	F	R	F	V	K	75
AAA	GCG	GCC	TCG	GAG	GTG	CTT	GTG	AAA	ACT	TTC	AGA	TTC	GTG	AAA	225
N	V	P	C	F	R	G	L	P	A	E	D	Q	L	R	90
AAT	GTG	CCG	TGT	TTT	CGG	GGT	TTG	CCG	GCG	GAA	GAC	CAG	CTC	CGG	270
L	V	R	N	S	W	A	P	L	L	V	L	G	M	V	105
CTT	GTG	CGC	AAC	AGC	TGG	GCG	CCG	CTG	CTG	GTT	TTG	GGC	ATG	GTG	315
Q	D	S	V	N	F	D	X	V	E	T	Q	Q	P	S	120
CAG	GAC	TCC	GTG	AAC	TTC	GAC	ANC	GTG	GAG	ACG	CAG	CAG	CCG	AGT	360
L	L	H	M	I	L	T	H	S	K	E	R	Q	Q	E	135
CTG	TTA	CAC	ATG	ATT	TTA	ACT	CAC	AGC	AAA	GAG	CGA	CAG	CAG	GAG	405
R	T	P	A	E	I	Q	G	P	G	V	P	V	S	D	150
CGC	ACT	CCG	GCA	GAG	ATC	CAG	GGC	CCA	GGG	GTG	CCG	GTC	AGT	GAC	450
A	E	G	I	K	M	F	Q	V	W	C	R	G	L	R	165
GCG	GAA	GGG	ATC	AAA	ATG	TTT	CAG	GTG	TGG	TGC	AGA	GGA	CTG	AGG	495
I	S	V	K	E	Y	A	F	L	K	G	A	I	L	F	180
ATC	AGC	GTC	AAA	GAA	TAC	GCG	TTT	CTG	AAG	GGA	GCG	ATA	CTG	TTC	540
T	P	V	T	E	L	E	C	R	E	Y	V	Q	A	L	195
ACC	CCG	GTG	ACG	GAG	TTG	GAG	TGT	CGG	GAG	TAT	GTC	CAG	GCG	CTT	585
Q	R	E	A	E	R	A	L	Y	E	H	V	R	T	V	210
CAG	AGA	GAG	GCT	GAG	CGC	GCG	CTT	TAC	GAG	CAC	GTG	AGG	ACG	GTC	630
H	R	G	K	A	G	R	F	G	R	L	R	A	V	L	225
CAC	CGG	GGT	AAA	GCG	GGCCGG	TTC	GGC	AGA	CTG	CGG	GCG	GTG	CTG		675
S	A	L	R	S	I	E	P	D	A	V	A	G	L	F	240
AGC	GCG	CTG	CGG	TCC	ATT	GAG	CCG	GAC	GCG	GTG	GCG	GGA	CTG	TTC	720
F	R	P	V	T	G	S	N	S	I	E	E	H	V	L	255
TTC	AGA	CCC	GTG	ACC	GGG	AGC	AAC	AGC	ATA	GAG	GAA	CAT	GTG	CTC	765
A	M	F	Y	G	R										261
GCT	ATG	TTT	TAT	GGG	CGG										783

sbSHP

M	D	N	G	C	H	C	S	A	E	S	L	S	N	P	15
ATG	GAT	AAC	GGG	TGT	CAT	TGT	TCA	GCC	GAA	AGT	CTC	TCT	AAT	CCT	45
I	L	F	K	I	L	S	Q	M	D	N	S	K	P	T	30
ATC	CTC	TTC	AAA	ATC	CTG	AGC	CAA	ATG	GAT	AAC	AGC	AAA	CCA	ACC	90
Q	N	G	F	N	Y	N	S	V	P	H	R	C	N	C	45
CAA	AAC	GGC	TTC	AAC	TAC	AAT	TCA	GTA	CCT	CAT	AGA	TGC	AAC	TGT	135
E	L	R	R	T	V	C	L	K	R	P	S	E	I	C	60
GAG	CTG	CGA	CGG	ACG	GTG	TGC	TTG	AAA	AGG	CCG	TCT	GAG	ATT	TGT	180
K	E	A	S	A	V	L	V	K	T	I	Q	F	M	K	75
AAA	GAA	GCT	TCA	GCA	GTT	CTG	GTT	AAA	ACC	ATC	CAG	TTC	ATG	AAG	225
N	L	P	A	F	N	Q	M	P	P	N	D	Q	F	S	90
AAC	TTA	CCT	GCT	TTT	AAC	CAG	ATG	CCA	CCA	AAC	GAC	CAG	TTC	TCA	270
L	L	K	S	C	W	A	P	L	F	I	L	G	L	A	105
CTT	CTC	AAA	AGT	TGC	TGG	GCA	CCG	CTC	TTC	ATT	TTG	GGC	CTG	GCC	315
Q	E	H	V	D	F	E	V	M	D	T	P	A	D	S	120
CAG	GAG	CAT	GTG	GAC	TTT	GAG	GTG	ATG	GAC	ACC	CCT	GCC	GAC	AGC	360
M	L	K	K	I	L	L	N	R	Q	E	S	S	D	M	135
ATG	CTG	AAA	AAG	ATT	CTC	CTG	AAC	CGC	CAG	GAG	AGC	TCC	GAC	ATG	405
E	R	D	Q	P	T	M	A	G	V	S	K	L	K	S	150
GAG	AGG	GAT	CAG	CCC	ACC	ATG	GCC	GGT	GTC	AGC	AAA	CTC	AAG	TCT	450
C	L	K	K	F	W	S	L	D	L	S	P	K	E	Y	165
TGC	CTT	AAA	AAG	TTT	TGG	AGT	TTG	GAT	TTG	AGT	CCA	AAG	GAG	TAT	495
A	Y	L	K	G	T	T	I	F	N	P	D	V	P	D	180
GCA	TAC	CTC	AAA	GGG	ACT	ACG	ATA	TTT	AAC	CCA	GAT	GTC	CCT	GAT	540
L	K	A	A	L	F	V	E	G	L	Q	Q	E	A	Q	195
TTA	AAG	GCA	GCT	CTG	TTT	GTT	GAA	GGC	TTG	CAA	CAG	GAA	GCT	CAG	585
H	A	L	R	E	V	V	Q	L	L	H	P	G	D	Q	210
CAC	GCA	CTC	AGG	GAG	GTC	GTA	CAG	CTC	CTT	CAC	CCA	GGG	GAC	CAG	630
E	R	F	A	R	V	L	L	T	A	S	M	L	Q	S	225
GAG	CGC	TTT	GCT	CGA	GTC	CTC	CTC	ACA	GCC	TCC	ATG	CTG	CAG	AGC	675
I	T	P	S	L	I	S	E	L	F	F	R	P	V	I	240
ATC	ACA	CCC	AGC	CTC	ATC	TCT	GAA	CTC	TTC	TTC	CGG	CCC	GTG	ATA	720
G	Q	A	D	L	L	E	L	L	V	D	M	L	F	C	255
GGC	CAG	GCT	GAT	CTG	CTG	GAG	CTG	TTG	GTT	GAT	ATG	CTC	TTC	TGC	765
R															256
AGG	TAG														771

Nucleotide sequence and deduced aminoacid sequence of sbDAX2 and sbSHP. Numbers on the right indicate aminoacid (top) and nucleotide (bottom) positions.

Chapter IV

***DAX1* promoter isolation and *in silico* characterization**

With: Heiner Kuhl, Richard Reinhardt, Deborah M. Power and Adelino V.M. Canário. Manuscript in preparation.

Abstract

DAX1 gene has been identified in three fish species. Earlier studies have suggested that this gene is involved in early larval development and later in gonadal and adrenal function as described in mammalian. However, there is still no data on the regulation of *DAX1* transcription or function in fish. Thus, the aim of this study was to identify putative transcription factors (TFs) that may be involved in *DAX1* promoter regulation and to study the promoter conservation between fish and mammal. We have isolated a 1kb sequence upstream the transcription start site (TSS) of the sb*DAX1* coding region, containing two TATA boxes flanked either by a GC box or by a CAAT box, suggesting the presence of two alternative promoters (P1 and P2). P1 promoter is located closer to the TSS, and is closer to the 5'UTR of the cDNA clone isolated in previous studies in the testis. We have identified different putative binding sites in the sb*DAX1* promoter that have been previously found to be used in mammalian *DAX1* promoter regulation, namely ERR, Nanog, SF1, WT-1, GATA and TCF/LEF. In addition, putative binding sites for Oct3/4 and AP1 were also found. Interestingly, we have found a DRE box for the binding of a transcription repressor Ca^{2+} binding protein (DREAM) and a GAGA box that has been hypothesized to be involved in germ cell differentiation. The putative P1 promoter is highly conserved between fish species; however, upstream sequences vary considerably. Several promoter frameworks were identified within fish and mammal *DAX1* promoters composed of 3-5 TFs, in which the order, orientation and distance between individual TFs was conserved, suggesting conserved regulation of these promoters across taxa. We have further identified other vertebrate annotated promoter genes that contain one or more of these frameworks, allowing the definition of 4 putative co-regulated networks within which, the *DAX1* gene is possibly involved. Thus, promoter conservation suggests a conserved role of vertebrate *DAX1* genes in early embryonic development, regulation of steroidogenesis, liver formation and metabolism, and bone and cartilage development. Altogether, the data suggests that despite sequence divergence of vertebrate *DAX1* genes, the promoter sequences have a highly conserved architecture, further suggesting that the regulation of *DAX1* is likely conserved throughout evolution.

4.1 Introduction

The embryonic development of gonads is a relatively conserved process among vertebrates. In mammals, different genes have been identified as being involved in the early events of primordial germ cell (PGC) formation, migration and proliferation in the urogenital ridge (UGR) (e.g. *Oct 3/4*, *c-kit*, *BMP4*) (see Saiti and Lacham-Kaplan, 2007 for review). Following migration of PGCs into the germinal ridge, cell division occurs to form oogonia and spermatogonia, and differentiation and migration of somatic cells is initiated concomitant with this process. Migration and differentiation of these cells lines lead to the activation of different gene cascades (e.g. *Wt-1*, *SF1*, *Lim9*, *Emx2*, *GATA-4*, *DAX1*, among others) that will be involved in the establishment of a bipotential gonad (see Wilhelm and Koopman, 2006 for review). Upon the activation of the male sex determining gene *SRY*, the bipotential gonad will start to differentiate as testis, while its absence directs activation of gene cascades towards the development of an ovary (Swain et al., 1998).

In fish germ cells and somatic cells are also clustered together and only at the later stages there is a clear distinction between these two different cell lines. The germ cells will develop into PGCs, which will ultimately lead to the establishment of gamete-forming cells within the gonad. Conversely, the somatic cells will lead to the development of structural and endocrine cells within the gonads. Upon the activation of specific gene cascades within these cells lineages, the two different cell types will ultimately lead to the development of either a female or a male gonad (see Devlin and Nagahama, 2002 for review). Most genes involved in mammalian sex determination, e.g., *DAX1*, *DMRT-1*, *SOX9*, and *WT-1*, are also found in fish, suggesting a conserved role throughout evolution (Kent et al., 1995; Kondo et al., 2002; Nakamoto et al., 2005).

DAX1 has been shown to be involved in sex determination and in adrenal formation and function. Human patients carrying *DAX1* mutations develop a broad spectrum of physiological problems beyond gonadal defects, as they also result in severe primary adrenal insufficiency involving deficient glucocorticoid and mineralocorticoid production, failure to respond to the elevated levels of ACTH (Achermann et al., 2001b) and in some cases, bone growth retardation (Seminara et al., 1999). Thus, it appears that *DAX1* is not only important for mammalian gonadal development but also for growth, metabolism and function of the hypothalamo-pituitary-adrenal-gonad (HPAG) axis. Moreover, recently, it has been discovered that the *DAX1* gene is expressed in skin and during osteoblast differentiation, tissues outside of the classical HPAG axis (Seminara et al., 1999). Altogether, it is clear that the role of *DAX1* in mammals is far from being determined.

In zebrafish, tilapia and European seabass, a *DAX1* homologue has been recently characterized (Wang et al., 2002, chapter 2; Zhao et al., 2006). *DAX1* mRNA levels have been shown to be expressed from 4 dph and during the sex determining period in sea bass (chapter 2) and tilapia, suggesting a role in this process (Wang et al., 2002). Gene expression analysis has also shown that, unlike in mammals, *DAX1* is ubiquitously expressed in most of the tissues of adult fish tested so far (Wang et al., 2002 and chapter 2), contrasting the typical HPAG axis distribution reported earlier in human and rodents. In chapter 2, a strong dimorphic pattern of expression of *DAX1* was also seen in most of the tissues. Male sea bass present higher *DAX1* levels than females in tissues from the digestive tract (liver, intestine and stomach), muscle, head kidney and in cartilage, while females express higher levels within the HPG axis than males. These results are quite similar to those obtained in tilapia, in which females presented lower *DAX1* mRNA levels than males in extra gonadal tissues (Wang et al., 2002). In addition, in zebrafish embryos, *DAX1* mRNA has been detected in head kidney, central nervous system, liver, brain and in trunk midline region between 4 and 32 days post fertilization (dpf), suggesting that the *DAX1* is involved in early embryonic development, well before gonads start to be developed (Zhao et al., 2006). Moreover, target disruption of *DAX1* in zebrafish resulted in impaired osmoregulation, suggesting that it has a determinant role in early adrenal function and glucocorticoid production in fish larvae (Zhao et al., 2006).

Several studies have shown the regulatory actions of *DAX1* gene in different gene promoters; however, few studies have focused on the regulation of the *DAX1* promoter. So far, the *DAX1* promoter has been found to be regulated by different transcription factors, namely by SF1, WNT signaling partners (beta catenin) and glucocorticoid receptor in mammals (Vilain et al., 1997; Gummow et al., 2003; Mizusaki et al., 2003). In fish, the *DAX1* promoter has never been isolated or characterized. Thus, the aim of this chapter was to isolate the sea bass *DAX1* promoter sequence, and to identify putative sequences for transcription factor binding sites (TFBS), in order to outline potential network partners for *DAX1* in fish. For this purpose, we have isolated the 1 kb putative promoter sequence of the sea bass *DAX1* and we have located putative TFBS that had been reported in mammalian *DAX1* promoters. Moreover, we have compared the promoter conservation between sb*DAX1*, mammalian promoters and other fish putative promoters, and have determined conserved putative gene networks within which *DAX1* may be involved in vertebrates.

4.2 Methods

4.2.1 Isolation of the sea bass *DAX1* promoter

The European sb*DAX1* gene structure has been characterized in chapter 2. A *DAX1* homolog gene was identified in the Stickleback genome by sequence similarity (www.ensembl.org). The genomic region containing the *DAX1* homolog gene in Stickleback was compared to the Sea bass genomic physical map obtained from end sequencing a sea bass BAC library (Whitaker et al., 2006). Sequencing and map construction was carried out by Heiner Kuhl, Alfred Beck and Richard Reinhardt (MPI Molecular Genetics, Berlin, Germany). The sea bass BAC clone spanning the homologous region to that of the Stickleback *DAX1* gene was shot gun sequenced and the corresponding contig sequence generated.

4.2.2 Identification of putative Transcription factor binding sites in the sb*DAX1* promoter

The identification of putative binding sites for basal transcription machinery (TATA box, GC box and CAAT box) on the *DAX1* promoter was done using the MatInspector software professional release 7.4.5 (Genomatix). Position weight matrices were used to represent the TFBSs using default parameters. The matrix family library Version 6.2 (Genomatix) was used for the analysis. Putative binding sites for transcription factors SF1, FOXL2, GATA, TCF/LEF, WT1 and LIM (Burris et al., 1995; Du et al., 2002; Mizusaki et al., 2003) were identified by visual inspection of the sb*DAX1* promoter sequence.

4.2.3 Identification of putative CpG islands in the *DAX1* promoter

Prediction of putative CpG islands in the *DAX1* promoter was done using the EMBOSS CpG plot software at Embl-EBI (www.ebi.ac.uk). CpG islands were identified as sequence fragments that had a total sum of C+G's higher than 50 % of the total DNA fragment and when the ratio of observed and expected G/C content was higher than 0.6.

4.2.4 *In silico* promoter analysis of vertebrate *DAX1* promoters

The putative 1 Kb promoter sequences of *DAX1* genes were retrieved from 2 fish species [tetradon (chr.2:8629001:8630000), fugu (Scaffold 42:1247946:1248946)] and 3

mammalian species [human (ChrX: 30237402:30238404), rat (Chrom. X: 73005301:73006300) and mouse (ChrX: 83436643: 83437643)] from genome databases (www.ensembl.org). The sequences (1kb upstream of the transcription start site) were compared to the *sbDAX1* promoter sequence by alignment (software Dialign, Genomatix) and by definition of a similar pattern (framework) of transcription factor binding sites (TFBSs) (software Frameworker, Genomatix). Position weight matrices were used to represent the TFBSs using default parameters. The matrix family library Version 7.0 (Genomatix) was used for the analysis. To define specificity and selectivity of the promoter sequences, the orthologous promoters were searched for two or more common TFBSs conserved in order, space (5-500 bp) and orientation. If found, the respective patterns of common TFBSs were tested on promoter libraries compiled from primate, rodent, other mammal and other vertebrate species (Genbank release 162) using the program ModelInspector from the Gems Laucher Suite (Genomatix). A framework is defined as a set of two or more TFBSs with a specific order, strand orientation, and distance range between the individual TFBSs.

4.3 Results

4.3.1 Putative *sbDAX1* promoter

We have isolated a 1 kb sequence upstream the TSS of the sea bass *DAX1* (Fig.1). Two putative TATA boxes were identified upstream of the first nucleotide of the initiation codon within this sequence. Close to the first and second TATA boxes, we have identified a putative GC box and a CAATT box, respectively. The first 116bp upstream the initiation codon corresponds to the 5'UTR of the *sbDAX1* transcript that has been previously identified in chapter 2.

[illegible]

Within this region we have identified a putative DRE and TCF/LEF binding site downstream the TATA box. Also, a (GA) microsatellite was found upstream the first TATA box, forming a long GAGA box and a WT-1 binding site. In addition, we have also identified duplicate putative binding sites for SF1 and a second binding site for TCF/LEF. Upstream the second TATA box, putative binding sites for Oct3/4, AP1, WT1 and GATA were also found (Table 1).

Table 1- Putative TF binding sites identified in Sea bass putative 1kb promoter. The putative sequence for TF binding site and promoter location are shown, together with the published consensus binding site and respective reference.

TF binding site	Consensus binding site	Sea bass sequence	Promoter Location	Reference
TCF/LEF1	(A/T)(A/T)CAA(A/T) G	TCAAAGA ATAAACA	-56/-49 bp -529/-523 bp	(Wegner, 199)
DRE	CCGGAGTCAAGG A	CCTCTGTCACTG	- 79/-67bp	(Ledo et al., 2000)
GC box	GGGCGG	GCGCGG	-145/ -150 bp	(Letovsky and Dynan, 1989)
GAGA box	(GA) _n	(GA) ₁₀ CA(GA) ₈ AGGGG G(GA) ₂	-263/ -207 bp	(Woodson et al., 1997)
SF1/ERR	AGGTCA TGGCCA	AGGTAA TGGCCT	-505/-500 bp -582/-576bp	(Yu et al., 1998a; Park et al., 2005)
TATA box	TATA(A/T)A(A/T)	TATAAAT TATAAAG	-590/ -586 bp -127/-123 bp	(Bucher, 1990)
Oct 3/4	ATGCAAAT	ATGACATCATAAT	-717/-693 bp	(Okumura-Nakanishi et al., 2005)
AP1	TGAGTC	TGAGTC	-726/ -718 bp	(Wisdom, 1999)
WT1	GCGTGGGCG(T/G) (G/T/A)(T/G) GAGAGGGGGAG	CGGTGGGTGGGT GAGAAGGGGGGAG	-780/-769 bp -194/ -207 bp	(Reddy and Licht, 1996)
CAAT box	XCAATX	GCAATT	-669/-665 bp	(Bucher and Trifonov, 1988)
GATA	(T/A)GATA(AG)	AGATAA	-858/-853 bp	(Ko and Engel, 1993)

The promoter region of the *DAX1* gene was found to be abundant in G + C content. In fact, CpG island analysis has yielded a predicted CpG island with 190 bp starting from -1 and ending at -190bp, flanking the downstream GAGA box. This CpG island contains 59 % GC and a ratio of observed and expected GC content of 0.6% (Fig. 2). Another proeminent feature of the *DAX1* gene is that the first coding exon is also rich in GCs and therefore has the same potential for methylation as the first 190 bp promoter sequence.

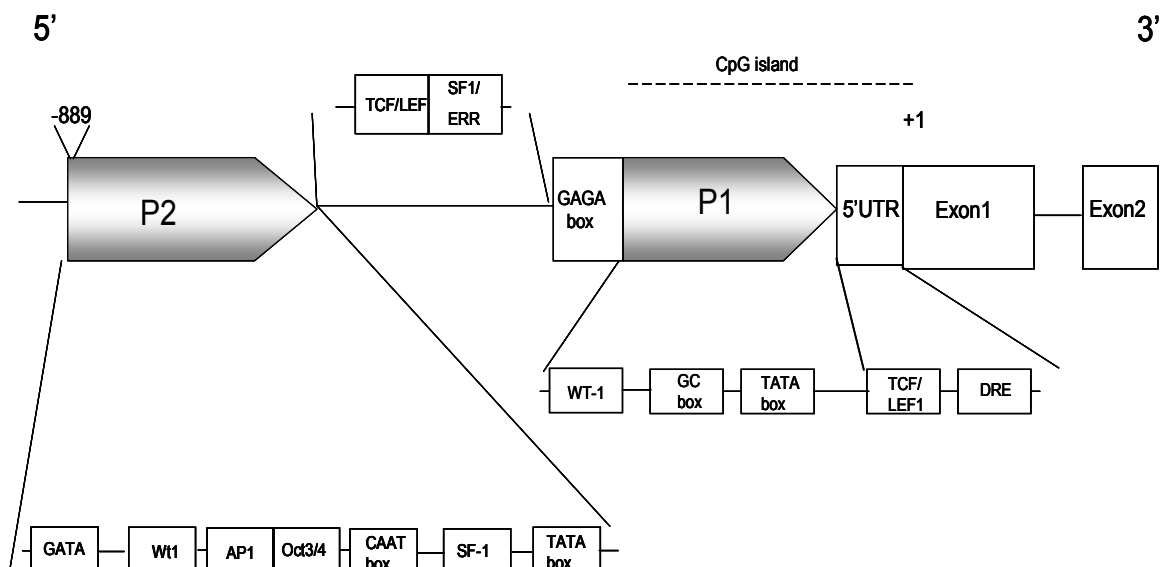


Figure 2- Sea bass *DAX1* gene structure and promoter region. Two putative promoters were identified in the 1 kb upstream sequence of sea bass *DAX1* gene (P1 and P2). Exons and introns spanning the coding region of *DAX1* gene are also shown. All transcription factors identified within the 1kb sequence are shown in the figure. The region containing a putative CpG island is represented by a dashed line. The transcription start site is marked as nucleotide +1, with all upstream sequence labeled with decreasing numbers, until -1000 bp. The putative TF binding sites identified in sea bass promoter sequence (for detail see table 1) are represented as square boxes.

4.3.2 Multiple Sequence Alignment of *DAX1* promoter in fish

The putative 1 kb *DAX1* promoter of fugu and stickleback were aligned in order to check the sequence conservation and also to compare the conservation of the putative binding sites for TFs identified in the sea bass promoter (Fig. 3).

The sea bass putative 1 Kb promoter region has 61% identity to that of the stickleback and 45% identity to that of fugu. Within this region, the sea bass 5'UTR and P1 promoter (-283 and -1 bp), has 72 % identity to the same region in the stickleback *DAX1* putative promoter and 66% identity to that of fugu. Also, within this region most putative binding sites found in the sea bass *DAX1* promoter are also present in the fugu and stickleback putative promoters. Thus, the presence of a TCF/LEF, DRE, TATA, GAGA box and WT-1 is conserved in the three fish species, although the GC box is absent from the fugu promoter. Interestingly, upstream the GAGA box, the promoter sequence of sea bass and stickleback are conserved but, the fugu promoter is not. Indeed, the putative binding sites for the other TFs identified upstream the GAGA box are highly

conserved between the stickleback and sea bass putative promoters, but are not present in the *fugu* putative promoter region.

* 20 * 40 * 60 * 80
 fugu_dax1_ : -----TATCAGTGTGTCCAGTAAAGTTGGAGCTGGCCTCAGCTCAGCTCTT : 52
 sea_bass_d : CTGACAATTAACCTCCACTGTAATCACCTCTGATTCCCTCTAGCCCCGTCCAGCTCTCTCTCTCAGCT-AGCAGCC : 79
 Sticklebac : ----- : -
 t t a t c c a c t a g c t g c t c a g t a g c

GATA

* 100 * 120 * 140 * 160
 fugu_dax1_ : CCTACTTTCTGTGCAAAATCATATAAATGTCCCTA-AAATAATTAAGCCTCAGATGTGTGTGGCCTATATTTTA- : 130
 sea_bass_d : AACCTAACCTGTGGTCCCTCTAGGAGCAATTAAGATAAACCTCTAATTCATCTGTTTACCCGACCTCTCTTACTCA : 159
 Sticklebac : -----TCCTTCCTAGGAGCAATTAAGATAAACCTCTAATTCATCTGTTTATCTGACCTCTCTTTTCCA : 65
 ct c tgtgtgcc TC TAggAgcaatTaagAtAAAcctcTAAtCaTcTcTtTatcCtGaCCTgTcTtctca

WT1

* 180 * 200 * 220 * 240
 fugu_dax1_ : --CCAGCTCTCTCTGATAAAGCAGCACTCCAGAAAGTCAAGTATGCTGGGAATAGTGTGGTCCCTTCC--TCACAAT : 206
 sea_bass_d : GTCTCTCATTCACT-GACCTCAGCTCCTCCCTCTCTTCCACCCAGCCCTCTTTCCTCCATCCTCTTTCAGTGACAGA : 238
 Sticklebac : TTCTCTCATCGCTTGACCTCAGCTCCTCCCTCTCTCT-----CCGCCCAACCACTCCCATCCTCTTTCAGTGACAGA : 140
 tCtctCaTccgcttGAcctcCActcCTcCC tc T ccaccCtc c ac cCcatcCtCcTtCagTgACAgA

* 260 * 280 * 300 * 320
 fugu_dax1_ : CAAAAACACGCAGGGTCTGTGGACTCTGTAAATTTGCTCTCTGAT---GAGCCTGAATTGGCCCACTACTTTTGT : 283
 sea_bass_d : GGCAAGGAGAGCATTGAGTCATGACATCATAATTACCAGCTCCTGTTTCAGGGTTAAATCAATATCAGTGTGTCCGAG : 318
 Sticklebac : AGCAAGGAGAGCATTGAGTCATGACATCATAATTACCAGCTCCTGTTTCAGGGTTAAATCAATATCAGTGTGTCCGAG : 220
 gcAAggagaGCAttGagtcaTGacaTCaTaaTtcaccaGctcCTGtTttcacGgtTaaATcaatCagTgtgTccGag

* 340 * 360 * 380 * 400
 fugu_dax1_ : GTAACA--GGAAGTTTGGTTC-CAGTCTGGCAGGATGCTTGGTCCCAACGGGCCACAGTCGGC---TCATTGAAAT : 356
 sea_bass_d : TCAAGGTGGAGGTGGCCTTCACAGCTCAGCTCTTCTACTTCTTCTCATAGAAAATCATATAAATTTGCCCTAAAAT : 398
 Sticklebac : TCAAGGTGGAGGTGGCCTTC---GCTCAGTCTCCCTACTTCTTCTCATAGAAAATCATATAAATTTGCCCTAAAAT : 297
 tcAAggttGGAgGtTggccTTC caGctCaG tctcccTaCTTctTtCtcAtaGaaaAtcaTataaattgTCcTaaAAAT

TF1 **TATA box**

* 420 * 440 * 460 * 480
 fugu_dax1_ : AAGAAAAAGTAGAAAAAGT--TTGTATTCATTTTTTTTGAAT--ATTTTGCAAGTTGCACCAAGATGGAT--AAG : 429
 sea_bass_d : AATTAAGCCTTCAGATCCGTGCTTGGCTATATTTTTTACCGGCTCAGTCTGATAAACAGCACTGCCAGAGAGTCAAGG : 478
 Sticklebac : AATTAAGCCTTCAGATCCGTGCTTGAATATATTTTTT-CCGCTTCTCCTGATAAACAGCTCCGCAAGGAGTCAAGG : 376
 AAttAagccttcAGAtccGTcgtTgG cTatATTTTTT ccGGcttc gtcTgatAAacaGCaCcgCcAggGA tcaAgG

SF1 **TCF/LEF** →

* 500 * 520 * 540 * 560
 fugu_dax1_ : TGATTTACCGATTTTTTTGAAGTCTTCTTTTAAATGACATTTGTTCCCACT---TTTGATAAGTGAATACATATTT : 506
 sea_bass_d : TAATGCTGAGAATAGAACTGGGCTCTGATTCCTTGTCTCTTTTGCTCTCTC---TTTTCCTCTCTCTTACAAAT- : 553
 Sticklebac : TAATTCGAGAATAGAACTGGGCTCCGATTCCTTGTCTGACCTGCCCCCTAAGGCTCATTCCTCTCTCCCACAATT : 456
 TaATtcc agaATagaacTGggCTccgatTccTtgtcTGaCttTGcctCt c tTt ttc ttctcTc cAcAaTt

* 580 * 600 * 620 * 640
 fugu_dax1_ : TCTAAATGCCAGCTGAAAAAATGTGACTTTTAAATGATTTTTTTTCTAAACCTTTTAGTAATTTTGTCTT----- : 579
 sea_bass_d : -----CTG-TGTAACCTAACAGCTCTATTGAATTTTGCCTTTTGTATG-ATTAG-----ATTATPGT----- : 609
 Sticklebac : AATAACGCAACCG-TTAAATCAACCGGGCTCTTGACCTCTGCTTCATAGTGCATTTCACACGTCATGTGCATAGTC : 535
 taa C G TgaAA AAccggG CT TTgA gtTgctTtttttgatgcaTTtg a TtaTgtT

* 660 * 680 * 700 * 720
 fugu_dax1_ : -----TTAACAGT--TCATGAATAAT-----CATGATTCATTAATTGAACAATATGTTT-CATTTA : 635
 sea_bass_d : -----TTGAAGTTTT--TTTTAAATAGTG-----GTGTGCTTGAATATGGCAAGATATGTGTGTTT- : 666
 Sticklebac : CAGAGGTGTGCTTTGAATGTTTAAAGTTTAAAAAGTGTGAGAGAGACGACACACACACACACACACACACACAC : 615
 TTgAa tTT TtttAAatagTg g gtGactca a At gacaaatAtgt t cattta

4.3.3 Conserved transcription factor frameworks of fish and mammal putative *DAX1* promoters

In order to identify possible molecular networks within which sea bass *DAX1* might be involved, we used an *in silico* promoter approach. Thus, analysis of sea bass, fugu, tetraodon, mouse, rat and human 1kb *DAX1* promoter sequences have shown significant conservation in promoter modules for potential TF binding. We have identified 5 different frameworks composed of 3-5 putative binding sites that are conserved in fish and mammalian, and are conserved in order, space and orientation (Fig. 4). These conserved frameworks are composed of consensus binding sites for the following TF families: homeodomain TFs (V\$HOMF and V\$NKXH), factors with moderate activity to homeodomain consensus sequence (V\$HOXF), EGR/nerve growth factor induced protein C and related factors (V\$EGRF), MYT1 C2HC zinc finger protein (V\$MYT1), v-ERB and RAR-related orphan nuclear receptor alpha (V\$RORA), TATA binding protein factors (V\$TBPF) and Brn POU domain factors (V\$BRNF).

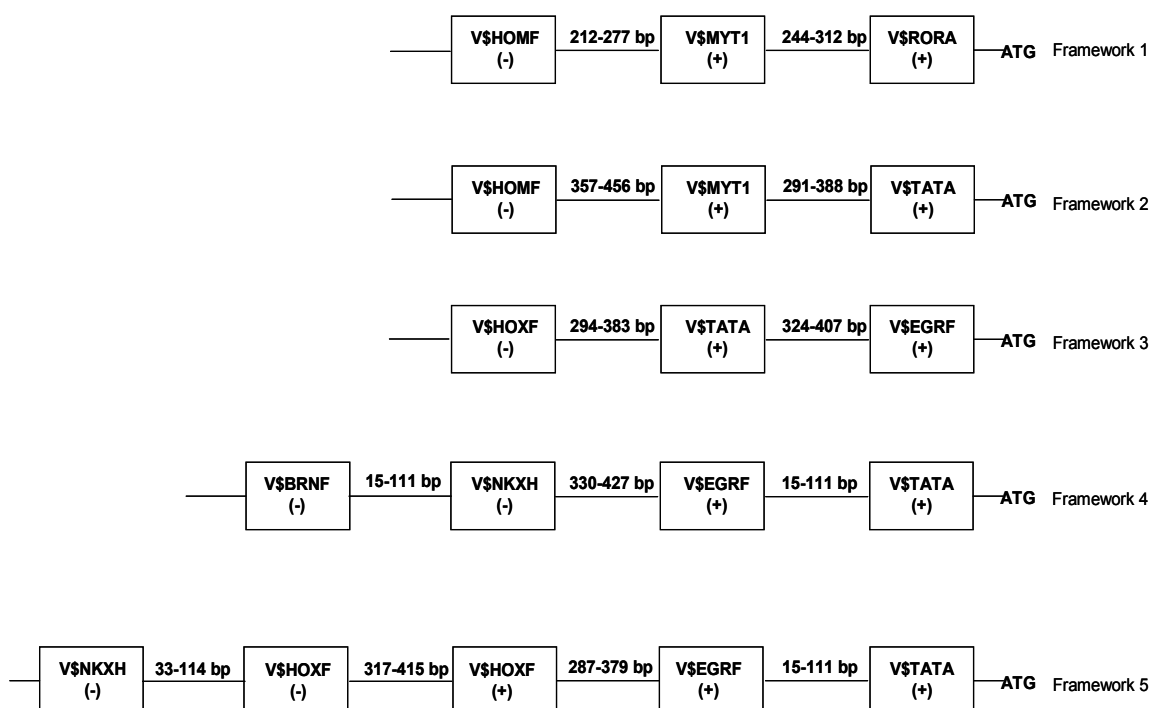


Figure 4- Conserved frameworks identified in fish and mammalian *DAX1* promoters.

Each framework is composed of 3-5 transcription binding sites, with conserved order, strand orientation and distance between TFs. The name of the TF family identified in *DAX1* promoters is shown within boxes. Within each box, the strand orientation is shown (+) or (-). The distance between each TF binding site is shown between each TF box. The codon ATG marks the beginning of the open reading frame, for orientation.

From each TF family identified in the conserved frameworks, the sea bass putative 1kb promoter contained conserved consensus sequences for homeodomain factors family members engrailed 1 (EN1), S8 and Nkx 2.5, for HOXF family members Nanog, Barx2 and Hox1, EGRF family member EGR1, MYT1 family member MYT1, RORA family member Rev-ERBA, TBPf family members TATA and ATATA binding proteins and BRNF family members Brn2.

4.3.4 Identification of gene promoters with conserved promoter frameworks as vertebrate *DAX1* promoters.

The conserved frameworks identified in the fish and mammalian *DAX1* putative promoters were compared to all annotated 5'UTRs and promoters in the Genbank release 162. We have identified 136 genes in vertebrates (mammalian and non mammalian) that also contain 1 or more of the frameworks identified in *DAX1* promoters. The identified genes contain identical frameworks that are composed of the same group of TF binding sites, and within the same order and distance. Within this list, and considering the available literature and Gene Ontology annotation on each of the genes identified, these were separated into different groups: Peptides, Peptide Hormones, Transcription factors, Channels and transporters, Chaperones, Transmembrane receptors, Nuclear proteins, Growth factors, Enzymes and Binding proteins (Fig. 5) (For detailed information see Appendix 1).

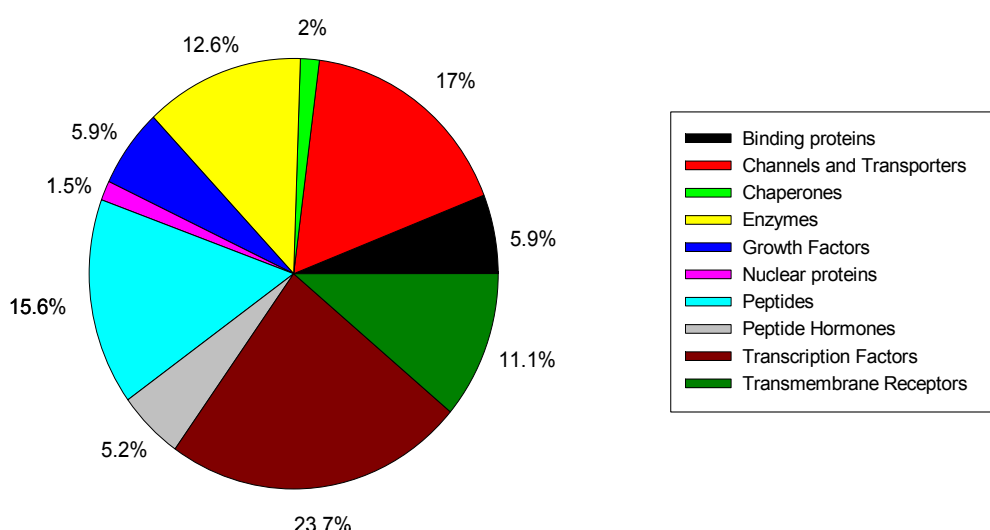


Figure 5- Pie chart representing the classes of annotated 5'UTR and promoter genes that contain one or more conserved frameworks as those identified in *DAX1* promoters. The percentage of each class of genes, relatively to the total number of genes identified with identical frameworks is shown in the graph.

The identified genes were then grouped into different clusters (I-IV), according to their role in a particular physiological process. Thus, Cluster I is composed of 18 genes that have been described as being involved in the appearance of primordial germ cells (PGC) in the urogenital ridge (URG), PGC migration, proliferation and differentiation into gonads (Table 2). Cluster II is composed of 44 genes that are involved in gonadal/adrenal development and in steroidogenesis (Table 3). Cluster III is composed of 47 genes that are involved in liver development and function, namely in sterol and bile acid production (Table 4). Cluster IV is composed of 48 genes that are involved in cartilage and bone development and homeostasis (Table 5).

Table 2- Cluster I: Group of genes with annotated promoter that contain the one or more frameworks in common with those identified in vertebrate *DAX 1* promoters, and that have been described as being involved in the urogenital and early gonad formation in mammalian. For each identified gene, the gene abbreviation, name description, function and literature are shown.

Gene	Description	Function	Literature
<i>Adamts</i>	Matrix metalloproteinase Adamts	Up regulated in male gonads in sex determination	(Beverdam and Koopman, 2006)
<i>AMH</i>	Anti-Müllerian hormone	Gonadal development	(Visser, 2003)
<i>BMP-4</i>	Bone morphogenic protein-4	PGC development and proliferation	(MacLaughlin and Donahoe, 2004)
<i>c-Fos</i>	Proto-oncogene c-fos	Germ cell survival	(Suomalainen et al., 2004)
<i>c-Kit</i>	Proto-oncogene c-kit	PGC development and proliferation	(Merchant-Larios and Moreno-Mendoza, 2001)
<i>COL IV</i>	Type IV collagenase	Involved in Leydig cell proliferation	(Yashwanth et al., 2006)
<i>Ctnnb1</i>	Beta-catenin	WNT signaling of the gonads	(Gummow et al., 2003; Parakh et al., 2006)
<i>EGF</i>	Early growth factor	Up regulated in early male gonad	(Beverdam and Koopman, 2006)
<i>FGF8</i>	Fibroblast growth factor 8	PGC development and proliferation	(McLaren et al., 2004)
<i>FGFR2</i>	Fibroblast growth factor receptor 2	PGC development and proliferation	(Bikfalvil et al., 1997)
<i>GATA-2</i>	GATA-2	Down regulated in ABP transgenic mice	(Petrusz et al., 2005)
<i>IGF1</i>	Insulin growth factor 1	PGC development and proliferation	(Nef et al., 2003)
<i>IGF2</i>	Insulin growth factor 2	PGC development and proliferation	(McLaren et al., 2004)
<i>IGF2R</i>	Insulin growth factor receptor 1	PGC development and proliferation	(McLaren et al., 2004)
<i>MMP2</i>	Metalloproteinase 2	AMH signaling in male	(Visser, 2003)
<i>MYF-5</i>	Muscle regulatory gene 5	Up regulated in ABP transgenic mice	(Petrusz et al., 2005)
<i>Pax2.1</i>	Paired box containing gene 2.1	PGC development and proliferation	(Capel, 2000)
<i>SF1</i>	SF1/Ad4BP	PGC development and proliferation	(Hiort and Holterhus, 2000)

Table 3- Cluster II: Group of genes with annotated promoter that contain the one or more frameworks in common with those identified in vertebrate *DAX 1* promoters, and that have been described as being involved in the HPAG axis function and development in mammalian. For each identified gene, the gene abbreviation, name description, function and literature are shown.

Gene	Description	Function	Literature
<i>11βHSDI</i>	11-beta hydroxysteroid dehydrogenase type I	Steroidogenesis	(Tomlinson et al., 2004)
<i>17βHSDI</i>	17 beta-hydroxysteroid dehydrogenase	Steroidogenesis	(Mindnich et al., 2004)
<i>3α HSD III</i>	3 alpha hydroxysteroid dehydrogenase III	Steroidogenesis	(Payne and Hayes, 2004)
<i>Agrp</i>	Agouti-related protein precursor	Regulation of Adrenal function and HPG axis	(Stanley et al., 1999; Mirabella et al., 2004)
<i>AKAP 79</i>	cAMP-dependent protein kinase	CaM signaling	(Colledge and Scott, 1999)
<i>AMH</i>	Anti-Müllerian hormone	Gonadal development and function	(Visser, 2003)
<i>AR</i>	Androgen receptor	Gonadal development and function	(Hiort and Holterhus, 2000)
<i>Calbindin D-9k</i>	Calcium binding protein D9K	Female reproductive tissues	(Choi et al., 2005)
<i>CaM</i>	Calmodulin	Regulation of steroidogenesis	(Leung and Steele, 1992)
<i>CaSR</i>	Calcium-sensing receptor	Functional and high expression in somatic ovarian cells	(Dell'Aquila et al., 2006)
<i>Cav1.2</i>	L-type calcium channel alpha 1c gene	Fertility	(Benoff et al., 2007)
<i>CFTR</i>	Cystic fibrosis transmembrane conductance regulator	Fertility and steroidogenesis	(Panesar, 1999; Hihnala et al., 2006)
<i>CR</i>	Calcitonin receptor	Steroidogenesis of the adrenals and gonads	(Esneu et al., 1996; Paul et al., 2008)
<i>CYP19</i>	Aromatase	Gonadal development and function	(Hiort and Holterhus, 2000)
<i>EGF</i>	Early growth factor	Up regulated in early male gonad	(Stocco et al., 2005; Beverdam and Koopman, 2006)
<i>ER beta</i>	Estrogen receptor beta	Gonadal development and function	(Hiort and Holterhus, 2000)
<i>ER alpha</i>	Estrogen receptor alpha	Gonadal development and function	(Hiort and Holterhus, 2000)
<i>FSHβ</i>	Follicle Stimulating Hormone subunit beta	Pituitary control of steroidogenesis	(Stocco et al., 2005)
<i>GC5</i>	OIGC5 gene for guanylyl cyclase	NOS/GC/GMP signaling pathway	(Krumenacker et al., 2001)
<i>GCAP1</i>	Guanylate cyclase activating protein 1	NOS/GC/GMP signaling pathway	(Krumenacker et al., 2001)
<i>Gcy</i>	Guanyl cyclase C	NOS/GC/GMP signaling pathway	(Krumenacker et al., 2001)
<i>Girk2</i>	G-coupled gated K ⁺ channel 2	Ar and PG signaling in the gonads	(Evaul et al., 2007)
<i>GnRH</i>	Gonadotropin-releasing hormone	Pituitary control of steroidogenesis	(Stocco et al., 2005)

Gene	Description	Function	Literature
<i>GnRHR</i>	Gonadotropin-releasing hormone receptor gene	Pituitary control of steroidogenesis	(Stocco et al., 2005)
<i>IGFBP5</i>	Insulin-like growth factor binding protein-5	Fertility	(Kelley et al., 1996)
<i>IGFBP6</i>	Insulin growth factor binding protein 6	Fertility	(Kelley et al., 1996)
<i>LH</i>	Luteinizing hormone beta subunit	Pituitary control of steroidogenesis	(Stocco et al., 2005)
<i>MaxiK</i>	MaxiK potassium channel beta subunit gene	Ca ²⁺ dependent K ⁺ channel in oocytes and sperm	(Nara et al., 1998; Munoz-Garay et al., 2001)
<i>MC1R</i>	Melanocortin-1 receptor	Expressed in testis	(Selz et al., 2007)
<i>NCX1</i>	Na ⁺ -Ca ⁺ exchanger	Sperm function	(Shiba et al., 2006)
<i>NHE3</i>	Na ⁺ /H ⁺ exchanger NHE3 isoform	Intracellular signaling	(Hihkala et al., 2006)
<i>Pkc α</i>	Protein kinase c-alpha	Down regulated in early male gonads	(Beverdam and Koopman, 2006)
<i>Pkc β</i>	Protein kinase C beta	Down regulated in early male gonads	(Beverdam and Koopman, 2006)
<i>POM</i>	Proopiomelanotropin	Pituitary control of steroidogenesis	(Szalay and Stark, 1982)
<i>PRL</i>	Prolactin	Pituitary control of steroidogenesis	(Stocco et al., 2005)
<i>PthrPR1</i>	Parathyroid hormone receptor 1	Probably mediating PthrP role in developing gonads and UGR	(Burton et al., 1990)
<i>SCN2A</i>	Voltage-gated sodium channel subtype II (Nav1.2)	Expressed in the testis	(Candenas et al., 2006)
<i>SCN7</i>	Sodium channel (Nax)	Expressed in male and female reproductive tissues	(Candenas et al., 2006)
<i>SF1</i>	SF1/Ad4BP	Gonadal development and function	(Merchant-Larios and Moreno-Mendoza, 2001)
<i>TR2 β</i>	Thyroid hormone receptor	Expressed in the gonads	(Nelson and Habibi, 2006)
<i>TSH β</i>	Thyrotropin beta-subunit	Pituitary control of steroidogenesis	(Gregoraszczuk and Ziecik, 1998)

Table 4- Cluster III: Group of genes with annotated promoter that contain the one or more frameworks as those identified in vertebrate DAX1 promoters, and that have been described as being involved in liver development and function. For each identified gene, the gene abbreviation, name description, function and literature are shown.

Gene	Description	Function	Literature
<i>3αHSD III</i>	3 alpha hydroxysteroid dehydrogenase III	Sterol and bile acid production	(Chiang, 2002)
<i>3βHSD III</i>	3 beta hydroxysteroid dehydrogenase III	Sterol and bile acid production	(Chiang, 2002)
<i>ABCA1</i>	ATP binding cassette transporter 1	Bile acid efflux	(Li and Glass, 2004)
<i>ABCB11</i>	ATP-binding cassette sub-family B member 11/bile salt export pump	Bile acid efflux	(Figge et al., 2004)
<i>ABCG1</i>	ATP-binding cassette transporter G1	Bile acid efflux	(Li and Glass, 2004)
<i>c-MET</i>	Proto-oncogene c-met (hepatocyte growth factor receptor)	Liver regeneration	(Huh et al., 2004)
<i>CYP7</i>	Cholesterol 7-alpha-hydroxylase	Sterol and bile acid production	(Chiang, 2002)
<i>EGF</i>	Early growth factor	Liver regeneration	(Komuves et al., 2000)
<i>ELF3</i>	Embryonic liver beta fodrin	Liver and bile duct development	(Mishra et al., 1999)
<i>FABP</i>	Brain-type fatty acid binding protein	Liver metabolism	(Erol et al., 2003)
<i>FGF2</i>	Fibroblast growth factor	Liver regeneration	(Rosenbaum et al., 1995)
<i>FGF8</i>	Fibroblast growth factor 8	Liver morphogenesis	(Lammert et al., 2003)
<i>FGFR2</i>	Fibroblast growth factor receptor 2	Liver regeneration and morphogenesis	(Rosenbaum et al., 1995)
<i>GATA-2</i>	GATA family member 2	Hematopoiesis	(Kim et al., 2004)
<i>GH</i>	Growth hormone	GH/IGF regulation of liver metabolism	(Davey et al., 2001)
<i>GHRBP</i>	Growth hormone receptor/binding protein	GH/IGF regulation of liver metabolism	(Gabrielsson et al., 1995)
<i>GHR-I</i>	Growth hormone receptor type I	GH/IGF regulation of liver metabolism	(Gabrielsson et al., 1995)
<i>HNF1a</i>	Hepatocyte nuclear factor 1alpha	Transcription regulators in liver	(Odom et al., 2004)
<i>HNF1b</i>	Hepatocyte nuclear factor 1-beta	Transcription regulators in liver	(Odom et al., 2004)
<i>IGF1</i>	Insulin growth factor 1	GH/IGF regulation of liver metabolism	(Davey et al., 2001)

Gene	Description	Function	Literature
<i>IGF2</i>	Insulin growth factor 2	Hematopoiesis	(Zhang and Lodish, 2004)
<i>IGF2R</i>	Insulin growth factor receptor 1	Liver development	(Zhang and Lodish, 2004)
<i>IGFBP5</i>	Insulin-like growth factor binding protein-5	Growth and metabolism	(Salih et al., 2004)
<i>IGFBP6</i>	Insulin growth factor binding protein 6	Growth and metabolism	(Gabillard et al., 2006)
<i>IkBα</i>	Ikappa B alpha gene	PPAR/Nfkb signaling in liver	(Li and Glass, 2004)
<i>IL-18</i>	Interleukin 18	Immune system	(Akira, 2000)
<i>IL-1b</i>	Interleukin 1 b	Immune system	(Mizuguchi et al., 1998)
<i>IL-8</i>	Interleukin 8	Immune system	(Huang et al., 1996)
<i>ILR-7</i>	Interleukin 7 receptor	Hematopoiesis	(Polli et al., 2005)
<i>LTBP-1L</i>	Latent transforming growth factor beta binding protein 1L	Liver regeneration	(Gong et al., 1998)
<i>MLST-1</i>	Liver specific transporter-1	Liver metabolism	(Choudhuri et al., 2000)
<i>NFKB2</i>	Nuclear factor kappa B subunit p100	PPAR/Nfkb signaling in liver	(Li and Glass, 2004)
<i>Pde4A</i>	cAMP-specific phosphodiesterase 4A	GH/GHR signaling	(Marchmont and Houslay, 1980)
<i>PGC-1 α</i>	Peroxisome proliferator activated receptor gamma coactivator 1 alpha	Liver metabolism	(Li and Glass, 2004)
<i>PPAR β</i>	Peroxisome proliferator activated receptor b	Liver metabolism	(Li and Glass, 2004)
<i>PPARγ</i>	CAST/Ei peroxisome proliferator-activated receptor gamma	Liver metabolism	(Li and Glass, 2004)
<i>PRL</i>	Prolactin	Regulation of liver function	(Buckley et al., 1988)
<i>P-type ATPaseII</i>	P-type ATPaseII /aminophospholipid translocase)	Heavy metal membrane transporter	(Halleck et al., 1998)
<i>RBPII</i>	Retinol binding protein II gene	Expressed in fish intestine and liver	(Liu et al., 2005)
<i>SLC10A2</i>	Ileal sodium-dependent bile acid transporter gene	Bile acid and sodium transporter	(Oelkers et al., 1997)
<i>TGF β</i>	Transforming growth factor beta III	Liver regeneration	(Bissell et al., 1995)
<i>TNFR2</i>	TNF receptor2	Liver regeneration and immune system	(Koerber et al., 2002)
<i>TTR</i>	Transthyretin	Liver function marker	(Schreiber, 2002)
<i>Ucp2</i>	Uncoupling protein 2 (mitochondrial proton carrier)	Thermogenesis and fatty acid production	(Tsuboyama-Kasaoka et al., 1999)
<i>UCP3</i>	Uncoupling protein 3	Thermogenesis and fatty acid	(Tsuboyama-Kasaoka

Gene	Description	Function	Literature
		production	et al., 1999)
<i>VEGF R2</i>	Vascular endothelial growth factor receptor 2	Liver development	(Lammert et al., 2003)

Table 5- Cluster IV: Group of genes with annotated promoter that contain the one or more frameworks as those identified in vertebrate *DAX1* promoters, and that have been described as being involved in the cartilage and bone formation. For each identified gene, the gene abbreviation, name description, function and literature are shown.

Gene	Description	Function	Literature
<i>Adamts</i>	Matrix metalloproteinase Adamts	Cartilage structure	(Tang, 2001)
<i>BMP-4</i>	Bone morphogenetic protein-4	Osteoblast differentiation	(Qi et al., 2003)
<i>CaM</i>	Calmodulin	Ca ²⁺ transport in skeletal tissues	(Lewinson and Boskey, 1984)
<i>CaSR</i>	Calcium-sensing receptor	Parathyroid function and regulation of bone formation	(Quarles, 2003)
<i>Cav1.2</i>	L-type calcium channel alpha 1c gene	Expressed in Osteoblasts and Osteoclasts	(Graf et al., 2005)
<i>Cdk2</i>	Protein kinase	Osteoblasts and osteoclasts differentiation	(Sunters et al., 1998)
<i>c-Fos</i>	Proto-oncogene c-fos	Osteoblasts and osteoclasts differentiation	(Sunters et al., 1998)
<i>CFTR</i>	Cystic fibrosis transmembrane conductance regulator	Bone ion metabolism	(Aris and Guise, 2005)
<i>CILP</i>	Cartilage intermediate layer protein	Cartilage protein	(Seki et al., 2005)
<i>c-Myc1</i>	Proto-oncogene c-myc-1	Chondrocyte proliferation and differentiation	(Farquharson et al., 1992)
<i>COL X</i>	Type X coll	Chondrocyte differentiation	(Vortkamp et al., 1998)
<i>COMP</i>	Cartilage oligomeric matrix protein	Cartilage protein	(Zaucke et al., 2001)
<i>CR</i>	Calcitonin receptor	Proliferation and maturation of chondrocyte	(Karsdal et al., 2006)
<i>Ctnnb1</i>	Beta-catenin	Osteoblast differentiation	(Westendorf et al., 2004)
<i>Cyclin D1</i>	Cyclin D1	Osteoblasts and osteoclasts differentiation	(Sunters et al., 1998)
<i>DSPP</i>	Dentin sialophosphoprotein precursor	Differentiation marker for odontoblasts	(Iohara et al., 2004)
<i>EGF</i>	Early growth factor	Induces differentiation of cartilage and bone tissue	(Urist et al., 1983)
<i>ETS1</i>	ETS1 proto-oncogene (FGF signaling)	Bone morphogenesis and tissue remodeling	(Vary et al., 2000)
<i>FGF2</i>	Fibroblast growth factor	Osteoblast development	(Marie, 2003)
<i>FGF8</i>	Fibroblast growth factor 8	Osteoblast development	(Marie, 2003)
<i>FGFR2</i>	Fibroblast growth factor receptor 2	Osteoblast development	(Marie, 2003)
<i>GH</i>	Growth hormone	GH/IGF signaling for growth	(Ohlsson et al., 1998)

Gene	Description	Function	Literature
<i>GHRBP</i>	Growth hormone receptor/binding protein	GH/IGF signaling for growth	(Zhou et al., 1997)
<i>GHR-I</i>	Growth hormone receptor type I	GH/IGF signaling for growth	(Ohlsson et al., 1998)
<i>GLUR2</i>	Glutamate receptor 2	Osteoclast resorptive activity	(Szczeniak et al., 2005)
<i>IGF1</i>	Insulin growth factor 1	Bone growth	(Naski et al., 1998)
<i>IGFBP5</i>	Insulin-like growth factor binding protein-5	Osteoblast proliferation	(Long, 2001)
<i>Igfbp6</i>	Insulin growth factor binding protein 6	Up regulated in cartilage degradation	(Meng et al., 2005)
<i>IkBa</i>	Ikappa B alpha gene	Osteoblast differentiation	(Qi et al., 2003)
<i>il-1b</i>	Interleukin 1 b	Bone resorption	(Kim et al., 2002)
<i>MID1</i>	Midline 1	Chondrogenesis induction	(Yates et al., 2001)
<i>MMP11</i>	Metallo matrix proteinase 11	Cartilage	(Davidson et al., 2006)
<i>MMP2</i>	Metalloproteinase 2	Cartilage	(Davidson et al., 2006)
<i>Nfkb2</i>	Nuclear factor kappa B subunit p100	Osteoclast differentiation	(Wagner and Matsuo, 2003)
<i>NHE3</i>	Na ⁺ /H ⁺ exchanger NHE3 isoform	Ion homeostasis in osteoblasts	(Mobasheri et al., 1998)
<i>Nurr1</i>	Steroid/thyroid hormone orphan nuclear receptor	Transcriptional regulator in cartilage and bone	(Lammi et al., 2004; Mix et al., 2007)
<i>Opn</i>	Osteopontin	Osteoblast differentiation	(Qi et al., 2003)
<i>Osteocalcin</i>	Osteocalcin	Osteoblast differentiation	(Qi et al., 2003)
<i>PGC-1 α</i>	Peroxisome proliferator activated receptor gamma coactivator 1 alpha	Stimulated by PTHRP in osteoblasts	(Nervina et al., 2006)
<i>PPAR β</i>	Peroxisome proliferator activated receptor b	Bone resorption	(Mano et al., 2000)
<i>PthrPR1</i>	Parathyroid hormone receptor 1	Bone remodeling / Osteoblast differentiation	(Naski et al., 1998; Qi et al., 2003)
<i>P-type ATPaseII</i>	P-type ATPaseII /aminophospholipid translocase)	Ion homeostasis in osteoblasts and osteoclasts	(Francis et al., 2002)
<i>Runx2</i>	Transcription factor CBFA1	Osteoblast differentiation	(Qi et al., 2003)
<i>SPARC</i>	Osteonectin	Bone and cartilage protein	(Brekken and Sage, 2000)
<i>TGF β</i>	Transforming growth factor beta III	Involved in bone and cartilage development	(Dieudonné et al., 1994)
<i>V-ATPase</i>	Proton pump ATPase (H ⁺ -ATPase)	Ion homeostasis in Osteoclasts	(Francis et al., 2002)
<i>VEGF R2</i>	Vascular endothelial growth factor receptor 2	Matrix remodeling	(Tombran-Tink and Barnstable, 2004)

4.4 Discussion

In the present study, we have isolated the 1 kb sequence, upstream the sea bass *DAX1* transcription start site (TSS). Within the promoter sequence, two TATA boxes were found suggesting the possibility of different promoter usage in *DAX1* transcription. In addition, the first 190 bp upstream the TSS contain the typical features of a CPG island as found in the human *DAX1* promoter (Zanaria et al., 1994), which suggests that *DAX1* transcription may also be regulated by methylation events. Interestingly, comparison of sea bass *DAX1* promoter sequence with those of mammalian species has also shown that most of the binding sites that are used in the regulation of mammalian *DAX1* transcription are also present in sea bass *DAX1*, further emphasizing that the regulation of this gene has been conserved throughout evolution. Thus, we have found different promoter binding sites that are used to regulate vertebrate *DAX1* transcription in different processes, namely in embryonic development, sex determination and early gonadal formation, steroid production, and also in novel processes like bone and cartilage formation and liver function.

DAX1 and embryonic development

The development of the gonadal, adrenal and urogenital systems is closely linked. Primordial germ cells (PGC) are destined to give rise to the entire population of mature germ cells of the organism. *Oct3/4* is a member of the POU homeodomain transcription factors, responsible for the regulation of pluripotency state of initial PGC (Ginis et al., 2004). Interestingly, *Dax1* has also been implicated in the maintenance of pluripotency of embryonic stem cells (ES). *DAX1* was found to be expressed in early pre implantation embryos as well as in ES cells (Clipsham et al., 2004). Differentiation of ES cells caused a decrease in the expression of *Dax1* similar to that observed for *Oct-4*. Conversely, disruption of the expression of *Dax1* by RNA interference as well as a conditional knockout in ES cells caused their differentiation (Niakan et al., 2006). Knockdown of *Dax1* using shRNAs led to a loss of pluripotence in ES cells. *Dax1* role in the maintenance of pluripotency has been further outlined since it was discovered that it interacted with Nanog, one of the *Oct3/4* partners in the maintenance of pluripotency of ES (Wang et al., 2006). Interestingly, we found a partially conserved binding site for *Oct3/4* and another for Nanog on sb*DAX1* promoter, suggesting that *DAX1* transcription is probably regulated by these transcription factors and also that the early expression of *DAX1* in sea bass (4dph) (see chapter 2) may be related to a specific function during the early embryonic development, as in zebrafish (Zhao et al., 2006). Further evidence for a role in early

embryogenesis arises from the ERR binding site identified within the *sbDAX1* promoter. ERR family (estrogen-related receptors) is composed of 3 orphan nuclear receptors that have been shown to involved in regulation of metabolism, energy, thermogenesis, growth and cell fate (Tremblay and Giguère, 2007). The three members bind to the same consensus binding site as SF1, and it has been shown that ERR γ regulates *DAX1* transcription in mammalian (Park et al., 2005). Moreover, ERR β has been shown to be involved in the maintenance of the pluripotency state of ES cells, and to interact directly with Nanog in ES cells (Wang et al., 2006). Altogether, identification of ERR, Oct3/4 and Nanog putative binding sites is consistent with a role of sea bass *DAX1* on early embryonic development and on the maintenance of pluripotency of ES cells at the early steps of gonadal and adrenal development, similar to the mammal *DAX1* homologue.

DAX1 and early gonadal development

PGC begin migrating into the embryonic mesoderm, and colonize the gonadal ridges. While migrating, they also proliferate and increase in number, where they become established and form an undifferentiated gonad. Several genes are known to be involved in the formation of the undifferentiated gonad. Among these genes two are determinant for the proper development of the bipotential gonad in fish and mammalian: *SF1* and *WT1*. *WT-1* is an upstream gene for both *SF1* and *DAX1* (reviews by Park and Jameson, 2005; von Hofsten et al., 2005). WT-1 and SF1 synergize to repress the transcription of *DAX1*, however, each of them can individually regulate *DAX1* transcription through direct binding to its promoter. Indeed, SF1 has been shown to bind the human *DAX1* promoter (Burris et al., 1995), and in murine promoter, duplicate binding sites for SF1 have also been shown to be used in *Dax1* regulation (Yu et al., 1998a). In the *sbDAX1* putative promoter, we have also found duplicate binding sites for SF1, with similar consensus sequences as found in murine promoter, suggesting that it may also regulate *DAX1* transcription in sea bass. Likewise, WT-1 is also able to activate *DAX1* transcription individually or through recruitment of co-activator FHL2 (Kim et al., 1999; Du et al., 2002). Interestingly, we have also found duplicate putative binding sites for WT-1 in *sbDAX1* promoter, suggesting that, similar to mammal *DAX1* gene, WT-1 may influence *DAX1* transcription. Altogether, these data suggest that *sbDAX1* may also be involved in early gonadal development and that its regulation throughout this process may be under the influence of WT-1 and SF1 as in mammalian. Further support for a role of fish *DAX1* in the establishment of the early gonad also arises from the promoter framework analysis of vertebrate *DAX1* promoters. Indeed, we have identified different genes that share identical promoter frameworks with those present in *DAX1* promoters and that are involved in PGC migration and early

establishment of the indifferent gonad. Within these genes, our analysis has identified *c-fos*, *c-kit*, *pax2.1*, *BMP-4*, *FGF* and *IGF* signaling cascades, which have been shown to be involved in the establishment of the early bipotential gonad in mammalian (Leung and Steele, 1992; Parker et al., 1999; Nef et al., 2003), suggesting that fish *DAX1* genes may also be involved within the gene networks that are responsible for germ cell migration and early gonad formation, as shown in mammalian species.

Interestingly, fish *DAX1* promoters, contain long (GA)_n microsatellites upstream the first TATA box, also present in mammalian *DAX1* promoters (although with smaller length), and named GAGA box. GAGA boxes are the consensus of the *Drosophila melanogaster* chromatin-remodeling factor, GAGA factor (dGAGA factor) (Soeller et al., 1993). Different studies have demonstrated that a GAGA box can be involved in transcription initiation with or without the presence of a functional TATA element. For example, in the *Drosophila hsp70* promoter, the GAGA box is required for TBP recruitment to its TATA motif (Weber et al., 1997). Alternatively, a number of GAGA boxes found in TATA- less promoters of other genes have been shown to be necessary for the initiation of transcription (Bossone et al., 1992). In addition, in mouse embryos, there is an accumulation of GAGA box-binding activity of maternal origin that later is also expressed at high levels in adult gonads. The abundance of GAGA box-binding activity in male and female gonads, and not in other adult tissues, has been suggested to indicate it plays a specific role(s), related to germ cell differentiation, in both oogenesis and spermatogenesis (Bevilacqua et al., 2000). Whether the GAGA box is used in the regulation of vertebrate *DAX1* transcription it is still not known. Nonetheless, its involvement in mammalian early embryogenesis and latter expression in gonads is coincident with *DAX1* roles described to date, and also support the view of a role in germ cell differentiation and early gonad formation.

DAX1 and sex determination

In mammals, activation of *SRY* within the male genital ridge initiates testis development. *SRY* is thought to trigger differentiation of the Sertoli cell lineage in the testis, which in turn directs the differentiation of the rest of the cell types in the testis. Thus, the lack of activation of this gene, fails to set Sertoli cell fate, and the cell will inevitably become a follicle cell (Swain et al., 1998). *Wnt4* is a gene whose activity antagonizes *SRY*: gene-disrupted mice displayed abnormal expression of both Leydig and Sertoli cell marker genes, which are normally not expressed in wild type fetal and neonatal ovaries, respectively (Vainio et al., 1999). The *WNT* genes encode a family of highly conserved glycoproteins that have been implicated in a variety of embryologic programs. *WNT* ligands bind to cell-surface Frizzled receptors, initiating a cytoplasmic signaling cascade

that results in beta-catenin stabilization, cytosolic accumulation, and subsequent translocation to the nucleus. Within the nucleus, beta-catenin classically complexes with a member of the T-cell Factor (TCF) family of transcription factors to activate target-gene transcription (Mulholland et al., 2005). It has been shown that upon Wnt4 stimulation, beta-catenin is able to activate *DAX1* transcription either through direct interaction with the TCF/LEF binding sites on its promoter or through direct interaction with SF1 on the *DAX1* promoter, in a TCF/LEF independent process (Mizusaki et al., 2003). Interestingly, the seabass *DAX1* promoter has duplicated putative SF1 and TCF/LEF binding sites, suggesting that WNT/beta-catenin signaling may also regulate *DAX1* transcription either through TCF/LEF dependent or independent mechanisms. In addition, analysis of vertebrate promoter frameworks have also yielded evidence to support this hypothesis since both SF-1 and beta- catenin (Ctnnb1) have been shown to share identical promoter frameworks with *DAX1* promoters, suggesting that they are working within the same networks. Thus, although to date no *SRY* or functional similar gene has been identified in most fish species, the promoter analysis shown in this chapter strongly suggests that most of the sex determining genes involved in mammalian sex determination are also likely involved in fish sex determination, including the fish *DAX1* gene.

DAX1 and the hypothalamus-pituitary-adrenal-gonadal axis

AHC, is characterized by adrenal insufficiency. Thus, plasma concentrations of mineralocorticoids and glucocorticoids are decreased and patients are usually unresponsive to ACTH stimulation (Kletter et al., 1991). HHG is also frequently found in patients with AHC (Prader et al., 1975; Muscatelli et al., 1994) and is detected by the failure of male patients to undergo puberty, as they also present low serum levels of FSH, LH and testosterone. Typically, FSH and LH do not respond to GnRH stimulation in these patients (Kletter et al., 1991). Interestingly, the deficit in pituitary hormones is selective for LH and FSH, as the production of other pituitary hormones (ACTH, GH, TSH, and PRL) is normal. Still, whether the HHG is a result of hypothalamic or pituitary dysfunction, or both, is not clear (Kletter et al., 1991). The human *DAX1* gene was cloned and identified as the gene responsible for both AHC and the associated HHG (Muscatelli et al., 1994; Zanaria et al., 1994). In patients with AHC who do not have large gene deletions, point mutations were found in the coding region of the *DAX1* gene, confirming that it was responsible for AHC. Thus, *DAX1* mutations lead to defective HPAG axis development and function. Interestingly, promoter analysis of vertebrate *DAX1* genes has identified a cluster of genes that have been shown to be involved in HPAG axis function and development. Within this cluster, we have included several pituitary hormones, namely *FSH*, *LH*, *GnRH*,

and *Proopio-Melanocortin* (*POM*), which are also affected in AHC and HHG syndromes. These results suggest that fish *DAX1* may also be involved in HPAG axis formation and/or function, as described in human *DAX1* gene.

In addition, *DAX1* has been shown to be a key regulator of steroid production in gonads and in adrenals. In fact, *DAX1* has been shown to function as a negative co-regulator of SF1 transactivation and is able to block overall steroid production at multiple levels of the steroid biosynthetic pathway of both the adrenal and gonadal tissues, e.g. *StAR* (Lalli et al., 1997), *CYP17* (Hanley et al., 2001), *CYP19* (Wang et al., 2001), *AMH* (Nachtigal et al., 1998), estrogens receptors α and β (Zhang et al., 2000) and androgen receptor (Holter et al., 2002). Interestingly, our promoter analysis has also identified most of these interacting partners as possible network partners of fish *DAX1* genes, namely, *AMH*, hypothalamic and pituitary hormones *GnRH*, *GnRHR*, *FSH β* , *LH*, *POM*, *PRL* and *TSH β* , the steroidogenic enzymes *CYP19*, *11 β HSDI* and *II*, *17 β HSDI*, *3 α -HSD III* and steroid receptors *AR*, *ER α* and *ER β* , suggesting that *DAX1* involvement in HPAG axis is most likely conserved from fish to mammals. The steroidogenic capacity of the gonads is under the tight control of pituitary hormones (reviewed in Stocco et al., 2005). Pituitary hormone stimulation of steroidogenesis is accomplished through the activation of different intracellular signaling pathways within the gonads, namely through the activation of Ca^{2+} -Calmodulin (CaM) (Gorczyńska and Handelsman, 1991), cAMP-PKA (Zhu and Birnbaumer, 1996), PKC (Nishimura et al., 2004) signalling pathways, upon stimulation. Interestingly, recent reports have focused on the role of CaM intracellular signaling on adrenal and gonad steroid production. Indeed, addition of calcium ionophores or of varying extracellular calcium concentrations to gonad cells of different fish species, elicits an increase in LH/hCG stimulated testosterone and 17 β -estradiol production (van der Kraak, 1991; Benninghoff and Thomas, 2005). Conversely, administration of a calcium chelator (EGTA), blocking L-type voltage sensitive calcium channels (VSCC) or blocking calcium release from intracellular stores via IP3R in gonad cells blocked the LH/hCG stimulated production of both testosterone and estradiol, suggesting that hCG effect on steroidogenesis is accompanied by increased calcium influx from extracellular compartments and remobilization of calcium from intracellular compartments (Benninghoff and Thomas, 2005; Benninghoff and Thomas, 2006). Furthermore, inhibition of CaM or CaM kinases (CaMK) also inhibits steroid production (Benninghoff and Thomas, 2005; Benninghoff and Thomas, 2006), further emphasizing the importance of the CaM signaling pathway in steroid production. Altogether, these data show that affecting the CaM messenger system or affecting Ca^{2+} influx or intracellular homeostasis, leads to profound changes in steroid capacity in endocrine cells.

Interestingly, our promoter analysis has yielded several genes that are involved in calcium homeostasis, namely calcium binding proteins (*calmodulin* and *calbinding*), *calcium sensing receptor*, *voltage gated calcium channel (Cav1.2)*, *Ca²⁺ dependent K⁺ channel (MaxiK)*, *Na⁺-Ca⁺ exchanger (Ncx1)*, among others, which suggests a link between Ca²⁺ intracellular signaling and regulation of *DAX1* transcription. Moreover, we have identified a Ca²⁺-binding protein response element or DRE response element that is used by the calcium binding protein DREAM. Indeed, DREAM is a nuclear Ca²⁺ sensor that upon binding to the DRE in basal conditions, results in transcriptional repression. DREAM functions as a homotetramer and its affinity for DNA is reduced upon binding to Ca²⁺ (Carrion et al., 1999). Furthermore, it was observed that the DRE element functions as a position-dependent, orientation-independent regulatory element that repressed transcription even when inserted in heterologous promoters. Importantly, the DRE functions only when located downstream from the TATA box, and because of this, this regulatory element was named as downstream regulatory element (DRE) (Ledo et al., 2000), as found in the *sbDAX1* promoter. Thus, this represents a direct mechanism for Ca²⁺-induced gene expression that is not dependent on changes in the activity of other transcriptional effectors through phosphorylation or protein: protein interactions (Carrion et al., 1999). Altogether, these results suggest that *DAX1* promoter might be under repressive actions of this nuclear Ca²⁺ sensor, and also suggests that vertebrate *DAX1* gene may be acting within the same gene networks as the different components of the CaM signaling pathway.

Thus, one of the possible scenarios in fish (and other vertebrates) is that the pituitary hormones stimulation of the adrenal and gonad cells, induces a shift in Ca²⁺ intracellular levels that activate the CaM intracellular signaling, which would lead to increased steroid enzyme activity and further steroid production. In this hypothesis, and considering that the *DAX1* protein has been shown to be a repressor of steroid production, activation of CaM signaling pathway would be counterbalanced by repression of *DAX1* levels, in order to enable steroid production. In this sense, one of the possible mechanisms to achieve this repression could be through enhanced binding of DREAM to *DAX1* promoter.

DAX1 and liver function

The third cluster of genes that we have identified in this promoter network has gathered several genes that have been shown to be involved in liver formation and function (cholesterol and bile acid production). To date, there is no data clearly demonstrating a role of *DAX1* in this pathway. In fact, liver function has been shown to be

under the regulation of another Nr0B family member, the *short heterodimer partner (SHP)* (Li et al., 2005). Interestingly, over the past years, several interactions have been found to occur between DAX1 and different genes involved in cholesterol production and function. One of these interactions occurs with SHP, with which DAX1 heterodimerizes in the cytoplasm and nucleus of mammal cells (Iyer et al., 2007). In addition, DAX1 is able to interfere with sterol regulatory element-binding proteins (SREBPs) and SF1 induction of a gene involved in the transport of cholesterol (High density lipoprotein receptor, HDL-R), thereby limiting the amount of substrate available for steroid hormone production (Lopez et al., 2001). In sea bass (chapter 2) and tilapia (Wang et al., 2002), high levels of *DAX1* mRNA expression are detected in both liver and intestine. Moreover, in zebrafish, *DAX1* is detected in liver of 3 dpf larvae, suggesting a role in early liver formation and development (Zhao et al., 2006). Thus, although no direct involvement of *DAX1* has been described in either liver formation or function, plenty of data is accumulating suggesting that *DAX1* may have a role outside the HPAG axis, namely in the liver.

DAX1 and bone/cartilage formation

Finally, a cluster was identified relating to cartilage and bone formation and homeostasis. To date, DAX1 has never been shown to play any role on cartilage or bone growth. However, recently, *DAX1* expression was found to increase with osteoblast cell differentiation in a transcriptional profiling study (Billiard et al., 2003). Moreover, in sea bass, one of the tissues with the highest *DAX1* mRNA expression is the gill cartilage (see chapter 2). In addition, recently, some reports on AHC due to *DAX1* mutations have found different patients presenting altered bone growth, (Rodríguez et al., 2006), even though GH levels were apparently normal. The relationship to calcium metabolism described above and the reported involvement of FSH in bone remodeling (Sendak et al., 2007) further supports a role in cartilage and bone formation and/or metabolism.

In summary, we have isolated a 1kb sequence upstream the TSS of the sb*DAX1* coding region that contains several features indicative of the existence of two putative promoter regions (P1 and P2). Within this promoter, we have identified a putative CpG island adjacent to the 5'UTR identified in the cDNA clone isolated in chapter 2. We have found different putative binding sites for transcription factors involved in pluripotency of ES (Oct3/4, Nanog and ERR), in primordial germ cell formation in the UGR and early undifferentiated gonad (SF1 and WT-1, GAGA box) and also in activation of gonadal differentiation (Wnt signaling, TCF/LEF, GATA). In addition, we have found a DRE box for the binding of a transcription repressor Ca^{2+} binding protein (DREAM), further suggesting

the involvement of Ca^{2+} signaling pathway in *DAX1* promoter regulation. The conservation of different promoter frameworks composed of 3-5 TFs within fish and mammal *DAX1* promoters suggests a conserved regulation of these promoters. In addition, the identification of these frameworks in other gene promoters and 5'UTRs annotated to date, allowed us to define 4 putative co-regulated networks within which the *DAX1* gene is possibly involved, namely in early embryonic development, regulation of steroidogenesis, liver formation and metabolism, and bone and cartilage development.

APPENDIX

Table 1- Gene promoters that share promoter frameworks with the vertebrate DAX1. Each gene is identified by its gene abbreviation, full name description and accession number as found in genebank database. The frameworks that are shared with the vertebrate DAX1 promoters are also shown. According to the Go gene ontology database, each gene was included into one of 10 classification groups: Binding proteins, Channels and transporters, Chaperone, Enzymes, Growth factor, Nuclear protein, Peptide, Hormone peptides, Transcription factors and Transmembrane receptors.

Gene	Description	Framework	Accession number(s)	Class
<i>IGFBP5</i>	Insulin-like growth factor binding protein-5	1	U20271	Binding proteins
<i>Igfbp6</i>	Insulin growth factor binding protein 6	2	AJ133763	Binding proteins
<i>LTBP-1L</i>	Latent transforming growth factor beta binding protein 1L	2	AF171934	Binding proteins
<i>RBPII</i>	Retinol binding protein II gene	2	AF338345	Binding proteins
<i>Calbindin D-9k</i>	Calcium binding protein D9K	2	L13042	Binding proteins
<i>GHRBP</i>	Growth hormone receptor/binding protein	3	AF120480	Binding proteins
<i>CaM</i>	Calmodulin	4	D90396	Binding proteins
<i>AKAP 79</i>	cAMP-dpendent protein kinase	1	M90359	Binding proteins
<i>Girk2</i>	G-coupled gated K ⁺ channel 2	1	AF040046	Channels and transporters
<i>SLC10A2</i>	Ileal sodium-dependent bile acid transporter gene	1	AF266724	Channels and transporters
<i>Kcnj6</i>	G-coupled gated K ⁺ channel 2	1/2	AF410425	Channels and transporters
<i>Kcnj13</i>	Kcnj13 gene for Kir7.1 potassium channel protein	1/3	AJ292748	Channels and transporters
<i>mlst-1</i>	Mlst-1 gene for liver specific transporter-1	1	AB037202	Channels and transporters
<i>TTR</i>	Transthyretin	1	AY505106	Channels and transporters
<i>Cav1.2</i>	L-type calcium channel alpha 1c gene	1/2	DQ187986/ L04569	Channels and transporters
<i>SLC34</i>	Sodium phosphate (Na/P)-cotransporter II	2/3/4	AJ003021/ AF058289	Channels and transporters
<i>ABCA1</i>	ATP binding cassette transporter 1	2	DQ142640	Channels and transporters
<i>CFTR</i>	Cystic fibrosis transmembrane conductance regulator	2	X95931	Channels and transporters

Gene	Description	Framework	Accession number(s)	Class
<i>MaxiK</i>	MaxiK potassium channel beta subunit gene	2	AF035045	Channels and transporters
<i>Ucp2</i>	Uncoupling protein 2 (mitochondrial proton carrier)	2	AF115319/ AB018416	Channels and transporters
<i>Nas1</i>	Sodium-sulfate cotransporter	2	AF199380	Channels and transporters
<i>Abcb11</i>	ATP-binding cassette sub-family B member 11/bile salt export pump	2/3	AF303740/ AY039785	Channels and transporters
<i>NCx1</i>	Na ⁺ -Ca ⁺ exchanger	2	U95138	Channels and transporters
<i>SCN2A</i>	Voltage-gated sodium channel subtype II	2	AF059683	Channels and transporters
<i>NHE3</i>	Na ⁺ /H ⁺ exchanger NHE3 isoform	3	U49386	Channels and transporters
<i>AbcG1</i>	ATP-binding cassette transporter G1	3	AF323643	Channels and transporters
<i>V-ATPase</i>	Proton pump ATPase (H ⁺ -ATPase)	3	AB045775	Channels and transporters
<i>SLC22A4</i>	Organic cation transporter SLC22A4	3	AB252052	Channels and transporters
<i>P-type ATPaseII</i>	P-type ATPaseII	3	AY775564	Channels and transporters
<i>SCN7</i>	Sodium channel	4	AY561435	Channels and transporters
<i>UCP3</i>	Uncoupling protein 3	4	AF127916	Channels and transporters
<i>HSP90</i>	Hsp90 gene	3	X15028 [	Chaperone
<i>HSF2</i>	Heat shock factor 2 gene	1/2	AF331667	Chaperone
<i>CYP19</i>	Aromatase	1/2/3	D50335/ DQ177458	Enzyme
<i>GC5</i>	OIGC5 gene for guanylyl cyclase	1/2	AB110697	Enzyme
<i>MMP2</i>	Matix metalloproteinase 2	1/2	AJ298926	Enzyme
<i>GUCY1A3</i>	Soluble guanylyl cyclase 1 alpha	1/2	AY034777/ AF327645	Enzyme
<i>3BHSD VI</i>	3 beta-hydroxysteroid dehydrogenase isomerase VI	2	AY046511	Enzyme
<i>CYP7</i>	Cholesterol 7-alpha-hydroxylase	2/3	U01962/ AF020317	Enzyme
<i>Cdk2</i>	Protein kinase	2/5	U07979	Enzyme
<i>INPP1</i>	Inositol polyphosphate 1-phosphatase	2	AF141325	Enzyme
<i>11BHSDI</i>	11-beta hydroxysteroid dehydrogenase type I	2	AY044083	Enzyme
<i>3alpha HSD III</i>	3 alpha hydroxysteroid dehydrogenase III	2	DQ379983	Enzyme
<i>Gcy</i>	Guanyl cyclase C	2	U20230	Enzyme
<i>Pde4A</i>	cAMP-specific phosphodiesterase 4A	3	AF142643	Enzyme
<i>Pkc alpha</i>	Protein kinase c-alpha	3	AF178928	Enzyme

Gene	Description	Framework	Accession number(s)	Class
<i>MMP11</i>	Metallo matrix proteinase 11	3	AF019253	Enzyme
<i>17BHSDI</i>	17 beta-hydroxysteroid dehydrogenase	3	M29037	Enzyme
<i>Adamts</i>	Matrix metalloproteinase Adamts	4	AY423552	Enzyme
<i>PkC beta</i>	Protein kinase C beta	5	D10022	Enzyme
<i>EGF</i>	Early growth factor	2	U57368	Growth factor
<i>BMP-4</i>	Bone morphogenic protein-4	2	AJ005076	Growth factor
<i>BMP5</i>	Bone morphogenic protein-5	2	S81957	Growth factor
<i>FGF8</i>	Fibroblast growth factor 8	2	U47009	Growth factor
<i>TGFbetalll</i>	Transforming growth factor beta III	3	AF537325	Growth factor
<i>IGF2</i>	Insulin growth factor 2	3	U71085	Growth factor
<i>IGF1</i>	Insulin growth factor 1	2	AF404761	Growth factor
<i>GH</i>	Growth hormone	2	X60419	Growth factor
<i>RAD17</i>	Cell cycle check point protein RAD17	1/2/4	AF106065	Nuclear protein
<i>SMC4</i>	Structural Maintenance of Chromosomes protein 4	2/5	AJ299717	Nuclear protein
<i>SPARC</i>	Osteonectin	1/2/3	U65081	Peptide
<i>DSPP</i>	Dentin sialophosphoprotein precursor	1	AF163151	Peptide
<i>PLN</i>	Phospholamban	2	M63600	Peptide
<i>IL-8</i>	Interleukin 8	2	M86923	Peptide
<i>Ccni</i>	Cyclin I	2	AF228739	Peptide
<i>COL X</i>	Type X coll	2/3/4	AF326473/ X73318	Peptide
<i>Ctnnb1</i>	Beta-catenin	2	AF486617	Peptide
<i>Opn</i>	Osteopontin	2/3	AY220127/ M84121	Peptide
<i>Selenoprotein</i>	Selenoprotein	2	M88462	Peptide
<i>GCAP1</i>	Guanylate cyclase activating protein 1	2/3	AF172707	Peptide
<i>il-1b</i>	Interleukin 1 b	2	AJ419848	Peptide
<i>Il-16</i>	Interleukin 16	2	AF121105	Peptide
<i>SPARCL1</i>	Matricellular related member SPACRC like 1	2	AF321976	Peptide
<i>CILP</i>	Cartilage intermediate layer protein	2/3	AB022430	Peptide
<i>IL-18</i>	Interleukin 18	2/4	AB095532	Peptide
<i>COL4A</i>	Collagen 4A	3	AF128530	Peptide
<i>MID1</i>	Midline 1	3	AY112909	Peptide
<i>Agrp</i>	Agouti-related protein precursor	3/4	AJ555493	Peptide

Gene	Description	Framework	Accession number(s)	Class
<i>IL-9</i>	Interleukin 9	3	M86593	Peptide
<i>Cyclin D1</i>	Cyclin D1	3	Z29078	Peptide
<i>COMP</i>	Cartilage oligomeric matrix protein	4	AF069520	Peptide
<i>TSHbeta</i>	Thyrotropin beta-subunit	2	M22739	Peptide hormone
<i>GnRH</i>	Gonadotropin-releasing hormone	2	X62651	Peptide hormone
<i>LH</i>	Luteinizing hormone beta subunit	2	EF194763	Peptide hormone
<i>POM</i>	Proopiomelanotropin	2	AB206465	Peptide hormone
<i>AMH</i>	Anti-Mullerian hormone	2	AM232702	Peptide hormone
<i>PRL</i>	Prolactin	1	AB162005	Peptide hormone
<i>FSHbeta</i>	Follicle Stimulating Hormone subunit beta	1	AF325218	Peptide hormone
<i>AR</i>	Androgen receptor	2	L15617	TF
<i>PPARGC1A</i>	Peroxisome proliferator activated receptor gamma coactivator 1 alpha	1/2	AY547550	TF
<i>Nfkb2</i>	Nuclear factor kappa B subunit p100	1	AF135125	TF
<i>GATA-2</i>	GATA family member 2	1	AB007371	TF
<i>Rev-ERB</i>	Rev-erb alpha gene	1/2/3	AY336126	TF
<i>Hnf1b</i>	Hepatocyte nuclear factor 1-beta	1/2	AY351674	TF
<i>Hnf1a</i>	Hepatocyte nuclear factor 1alpha	1/2	AY517630	TF
<i>SRY</i>	Sex determining gene in Y chromossome	2	AF026566	TF
<i>IkBa</i>	IkappaBalpha gene	2	Z30209	TF
<i>Runx2</i>	Transcription factor CBFA1	2/3	AF155360	TF
<i>Eralpha</i>	Estrogen receptor alpha	2	AF169237	TF
<i>PPARb</i>	Peroxisome proliferator activated receptor b	2/3	AF329818/ AF187850	TF
<i>Tcfap2c</i>	Transcriptional factor AP-2 gamma	2	AY196820	TF
<i>PPARG</i>	CAST/Ei peroxisome proliferator-activated receptor gamma	2	AY236530	TF
<i>PAX-7</i>	Transcription factor PAX7	2/3	AY328081/ AJ130875	TF
<i>Dmtf1</i>	Cyclin D-interacting myb-like transcription factor	2	AY702209	TF
<i>TR beta2</i>	Beta 2 thyroid hormone receptor	2	U15542	TF
<i>Nurr1</i>	Steroid/thyroid hormone orphan nuclear receptor	2	U86783	TF
<i>c-Kit</i>	Proto-oncogene c-kit	2/4	X86451	TF
<i>SF1</i>	SF1/Ad4BP	2	AB017353	TF

Gene	Description	Framework	Accession number(s)	Class
<i>c-myc1</i>	Proto-oncogene c-myc-1	2	AB103397	TF
<i>ER beta</i>	Estrogen receptor beta	2	AF516875	TF
<i>pax2.1</i>	Paired box containing gene 2.1	2	AY024360	TF
<i>Pax6</i>	Paired box containing 6	2	AY048575	TF
<i>MYF-5</i>	Muscle regulatory gene 5	2	AY050251	TF
<i>FABP</i>	Brain-type fatty acid binding protein	2/3	AY145893/ X73318	TF
<i>FGF</i>	Fibroblast growth factor	2	AF019634	TF
<i>TTF-1</i>	Thyroid-specific transcription factor 1	2	AF027332	TF
<i>p54</i>	Transcriptional repressor p54	2/3	AF052744	TF
<i>ETS1</i>	ETS1 proto-oncogene	3	AF221504	TF
<i>ELF3</i>	Ets-related transcription f	3	AF287266	TF
<i>c-Fos</i>	Proto-oncogene c-fos	4	DQ298418	TF
<i>c-met</i>	Proto-oncogene c-met (hepatocyte growth factor receptor)	1/2/3	AF046925	Transmembrane receptor
<i>CR</i>	Calcitonin receptor	1/2	AJ271672/ F333472	Transmembrane receptor
<i>CaSR</i>	Calcium-sensing receptor	1	U20760	Transmembrane receptor
<i>GLUR2</i>	Glutamate receptor 2	2	AF250875	Transmembrane receptor
<i>Igf2r</i>	Insulin growth factor receptor 1	2	AJ249895	Transmembrane receptor
<i>TNFR2</i>	TNF receptor2	2	U39488	Transmembrane receptor
<i>Osteocalcin</i>	Osteocalcin	2	U66847	Transmembrane receptor
<i>GnRHR</i>	Gonadotropin-releasing hormone receptor gene	2	U92469	Transmembrane receptor
<i>GHR-I</i>	Growth hormone receptor type I	2	DQ899640	Transmembrane receptor
<i>VEGF R2</i>	Vascular endothelial growth factor receptor 2	2/3	EF621764	Transmembrane receptor
<i>PthrPR1</i>	Parathyroid hormone receptor 1	2	AJ550897	Transmembrane receptor
<i>MC1R</i>	Melanocortin-1 receptor	2	AB026663	Transmembrane receptor
<i>FGFR2</i>	Fibroblast growth factor receptor 2	3	AF233344	Transmembrane receptor
<i>ILR-7</i>	Interleukin 7 receptor	3	DQ821273	Transmembrane receptor
<i>ILR-8</i>	Interleukin receptor 8	3	U11866	Transmembrane receptor

Chapter V

Ca²⁺-Calmodulin signaling regulates androgen production in the testis of adult male Tilapia (*Oreochromis mossambicus*), *in vitro*.

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Manuscript in preparation.

Abstract

In fish, gonadotropin stimulated testosterone production has been shown to be regulated by Ca^{2+} -Calmodulin (CaM) signalling pathway. However, there is no information regarding a putative role in male fish steroidogenesis. The objective of this study was to determine whether CaM regulate, *in vitro*, the androgen production in tilapia testis. For this, testicular tissues were incubated with a CaM inhibitor, W7, and the incubation media were assayed for androgens, glucocorticoids and progesterone metabolites. *In vitro* incubations with 80 μM W7 for 6 hours decreased basal 11KT levels significantly ($p < 0.01$). Basal testosterone and androstenedione levels were also significantly decreased at the highest dose of W7 tested (80 μM), but in extended incubation periods (8 hours). On the contrary, only residual 11-deoxycortisol levels could be detected in control media but, after W7 treatment, the gonad started producing increased 11-desoxycortisol levels and also produced cortisol, suggesting that inhibition of CaM favours glucocorticoid and is detrimental to androgen production. Furthermore, levels of 17, 20 α -dihydroxy-4-pregnen-3-one in control and W7 media were similar, suggesting that the reduction of androgen levels were not due to an effect of this inhibitor at earlier steps of steroidogenesis. *In vitro* incubations with forskolin also demonstrated that androgen production is a target for cAMP regulation, as forskolin induced a significant increase in testosterone, 11-ketotestosterone and cortisol production in the gonads. Testicular incubations with or without calcium and with EGTA addition to the incubation medium did not affect androgen production, suggesting that the effect of W7 on androgen production is likely specific from the lack of CaM regulatory actions and not due to altered Ca^{2+} levels. Concomitant treatment with W7 and forskolin also showed that the addition of W7 blocked androgen stimulation by forskolin, suggesting that there is cross-talk between the two pathways in androgen production. Altogether, the results presented in this chapter show that there are two distinct signalling pathways regulating androgen production in tilapia testis, via cAMP-PKA and via CaM.

5.1 Introduction

The mammalian *DAX1* has been shown to be a key regulator of steroid production in gonads and in adrenals. Indeed, this gene has been shown to function as a negative co-regulator of SF1 transactivation and is able to block overall steroid production at multiple levels of the steroid biosynthetic pathway of both the adrenal and gonadal tissues (Lalli et al., 1997; Nachtigal et al., 1998; Hanley et al., 2001; Wang et al., 2001; Holter et al., 2002). Interestingly, our *DAX1* promoter analysis (see chapter 4) has also identified different steroid enzymes, steroid receptors and hormone genes (e.g. *CYP19*, *11 β HSDI and II*, *17 β HSDI*, *3 α HSD III*, *AR*, *ER α* and *ER β* , and *AMH*) that share common frameworks with those found in fish and mammalian *DAX1* promoters. We have also identified different hypothalamic and pituitary hormone genes known to be key regulators of steroid production in the gonads of mammalian and fish species (e.g. *GnRH*, *GnRHR*, *FSH β* , *LH*), and components of different signalling pathways used by these hormones to regulate steroid production in steroidogenic tissue, namely of the CaM signalling pathway (calmodulin, calcium and potassium channels and exchangers) (Gorczyńska and Handelsman, 1991; Benninghoff and Thomas, 2005; Benninghoff and Thomas, 2006). Altogether, these results suggest that *DAX1* is likely involved in the gene network regulating steroid production in fish, as demonstrated in mammals. In addition, in chapter 4 we have also identified in sb*DAX1* promoter a binding site for DREAM (a Ca²⁺ binding protein), further suggesting that in fish, the *DAX1* gene transcription is most likely affected by the same factors that modulate the Ca²⁺ levels in steroid producing cells, namely by pituitary hormones.

The role of brain and pituitary in the regulation of reproduction and steroidogenesis in vertebrates is well established. Gonadotropin releasing hormones (GnRH) produced in the brain control gonadotropin release from the pituitary which, in turn, has decisive actions on the sexual differentiation, gametogenesis and reproductive function. The regulation of synthesis of gonadal steroids by the pituitary gonadotropins FSH and LH (homologous to GtH I and GtH II in fish) has been shown to be mediated through binding to G-protein coupled receptors (GPCRs) which will lead to the activation of different transduction pathways, namely the Ca²⁺ messenger system. There is a strong body of evidence that GnRH, FSH, LH and hCG stimulation of steroidogenesis in mammalian gonads leads to an increase in intracellular calcium levels, either through release of intracellular stores or by mobilization of calcium from extracellular compartments (Sullivan and Cooke, 1986). Extracellular Ca²⁺ levels have been shown to be determinant for steroid production since omission of Ca²⁺ from the incubation medium in rat Leydig cell

cultures leads to reduced LH/hCG stimulation of steroid synthesis. This reduction is only abolished after addition of Ca^{2+} to the incubation medium (Manna et al., 1999). Furthermore, studies with calcium channel inhibitors have shown that LH/hCG-stimulated steroidogenesis in bovine, human and rat adrenocortical and granulosa cells is reduced when calcium influx is blocked (Moger, 1983; Rossier et al., 1996; Lotshaw, 2001; Agoston et al., 2004). In contrast, administration of a calcium channel agonist BAYK8644, induced steroidogenesis in adrenal cells (Manna et al., 1999). At the intracellular level, Ca^{2+} increases are accompanied by increased binding to calmodulin (CaM), a calcium binding protein that will ultimately activate several signaling molecules, transcription factors and enzymes involved in the control of steroid production. Studies with rat Leydig cells have shown that when Ca^{2+} and CaM are administered to cells via liposome, the LH stimulated steroidogenesis is potentiated, suggesting that this complex is involved in cholesterol transport into the mitochondria (Hall et al., 1981). Further information on Ca^{2+} -CaM involvement on mitochondria steroidogenesis was obtained from the administration of mitochondria Ca^{2+} uptake inhibitor, ruthenium red, which was effective in blocking steroid production in adrenal cells (Capponi, 1988). The CaM complex has also been shown to be involved in the regulation of steroidogenic enzymes. In fact, studies with calmodulin and Ca^{2+} -dependent calmodulin kinase (CaMKs) inhibitors have shown that -CaM signaling pathway regulates the activity and/or transcription of 17β -HSD (the enzyme responsible for the conversion of androstendione into testosterone), of P450 scc (responsible for cholesterol cleavage and pregnenolone production) and of 11β -HSD (involved in glucocorticoid production) (Khanum et al., 1997; Jayes et al., 2000; Ge and Hardy, 2002). Recently, several studies have also shown that calmodulin interacts physically with steroid receptors, androgen receptor and estrogen receptor α , unravelling another regulatory step to steroid signalling (Garcia-Pedrero et al., 2002; Cifuentes et al., 2004; Li et al., 2005).

In fish, few studies have searched for a role of CaM in steroidogenesis. In goldfish, administration of the calcium ionophore A23187 to ovarian cells indicated that calcium had an effect on steroidogenic capacity, although it pointed for different sites of action when compared to other vertebrate species (Veldhuis et al., 1984; van der Kraak, 1991). In Atlantic croaker, addition of calcium ionophore A23187 to ovary incubations increased basal and hCG stimulated testosterone and estradiol production in a dose-dependent manner (Benninghoff and Thomas, 2005). Studies with varying extracellular calcium levels failed to establish any correlation with basal testosterone levels in both goldfish and Atlantic croaker ovarian cells (Veldhuis and Klase, 1982; Veldhuis et al., 1984; van der Kraak, 1991; Benninghoff and Thomas, 2005), although in the latter species it potentiated the hCG stimulated testosterone and estradiol production. Administration of a calcium

chelator (EGTA) to the Atlantic croaker ovarian cells effectively blocked the hCG stimulated production of both testosterone and estradiol, suggesting that hCG effect on steroidogenesis is calcium dependent (Benninghoff and Thomas, 2005). Furthermore, administration of different L-type voltage sensitive calcium channel (VSCC) inhibitors to ovarian cells have shown that both basal and hCG stimulated accumulation of testosterone and estradiol is also blocked, suggesting that steroid production involves calcium influx through L-type VSCC (Benninghoff and Thomas, 2005; Benninghoff and Thomas, 2006). Additionally, administration of TMB-8, an inhibitor of calcium release from intracellular stores via IP3R, was also shown to be effective in blocking hCG stimulation of testosterone accumulation in ovarian cells, suggesting that hCG stimulation also involved intracellular Ca^{2+} remobilization from intracellular compartments (Benninghoff and Thomas, 2005). Similar to the involvement of calcium influx and intracellular calcium mobilization in hCG stimulated steroidogenesis, both CaM inhibitors (W-7 and TFP) and CaMK inhibitors (KN-62) have also been shown to block accumulation of testosterone in the Atlantic croaker ovarian cells (Benninghoff and Thomas, 2005; Benninghoff and Thomas, 2006). Altogether, these studies show that gonadotropin stimulated testosterone production in the ovary requires an increase in intracellular Ca^{2+} levels, via activation of VSCC and IP3Rs, and the involvement of Ca^{2+} -CaM complex in the activation of CaMKs.

The work done in the Atlantic croaker has shown that modulation of CaM levels can affect steroid production in the ovaries, but to date, there is still no data on fish steroid regulation in testis and on whether this influence is affecting the *DAX1* gene transcription as suggested in chapter 4. In order to investigate these two aspects, we have used testis from Mozambique tilapia (*Oreochromis mossambicus*) in an *in vitro* system. Male tilapias are territorial and social behavior, both aggression and courtship is correlated to androgen levels (Oliveira et al., 1996; Borges et al., 1998). Moreover, in this species strong correlations have been found between androgen levels (Testosterone and 11-Ketotestosterone) measured after group formation and the acquired social status (Oliveira et al., 1996). Interestingly, group formation and the establishment of social status amongst male tilapias is achieved in one day (Oliveira et al., 1996), suggesting that there is the activation of a regulatory mechanism that has severe influences on androgen levels, within a very short time frame. Thus, the objective of this chapter was to establish whether steroid production in testis Mozambique tilapia is modulated by CaM and whether modulation of these levels are mediated by changes in *DAX1* transcriptional levels.

5.2 Methods

5.2.1 General tissue dissection

Mature male tilapias ($n=6$ or 7) were transferred to holding tanks and allowed to acclimate for 2 weeks. At the start of the experiment fish were anaesthetized with a lethal dose of 2-phenoxyethanol (1:10000 2-phenoxyethanol: seawater; Sigma-Aldrich, Madrid, Spain) and weighed. Testicular tissue from 7 fish were removed and transferred immediately to ice cold Cortland medium (pH=7.8) containing 118.5 mM NaCl, 4.7 mM KCl, 1.2 mM NaH_2PO_4 , 10 mM NaHCO_3 , 1.5mM CaCl_2 and 1.5 mM MgSO_4 supplemented with 5mM glucose, 15 mM Hepes, 60mg/L penicillin and 100 mg/L streptomycin. The incubation medium was saturated with a 99% O_2 and 1% CO_2 mixture before the beginning of each experiment. Gonads from each fish were cut into small fragments with approximately the same weight. Fragments from each individual gonad were transferred to 48-well plastic tissue culture plates containing 1ml incubation media. Each treatment consisted of 6-7 wells ($n=6-7$), each containing 3 fragments from a different fish, so that all fish were assayed for all experimental conditions. Details on the *in vitro* experimental incubation procedures are described below for each experiment.

5.2.2 The effect of extracellular calcium and of calmodulin inhibition on testicular basal steroid production.

In order to study the effects of extracellular calcium on basal steroid production, testicular fragments from 7 fish were incubated for 6 hours in 1 ml of either Cortland medium, Cortland medium without calcium or Cortland medium containing the calcium chelator EGTA (1 mM). The fragments were incubated for 6 hours, after which the incubation media was collected and stored at -20°C until assayed.

The effect of calmodulin (CaM) inhibition on basal steroid production was also studied, using a CaM inhibitor W7 [N-(6-aminohexyl)-5-chloro-1-naphtalenesulfonamide]. Similar to that described in calcium incubations, testicular fragments from 7 fish were incubated for 6 hours in 1 ml Cortland medium containing two different concentrations (20 μM or 80 μM) of W7 dissolved in 10% DMSO and 150 mM NaCl or in Cortland medium containing the same amount of 10% DMSO and 150 mM NaCl but without the calmodulin inhibitor (control group). After the incubation the media were collected and stored at -20°C until assayed for steroid content.

5.2.3 The effect of forskolin and W7 on androgen production.

In order to study the effects of adenylyl cyclase stimulation on basal steroid production, we have studied the effect of forskolin in basal steroid production. Thus, we have done a preliminary experiment, where by testicular fragments from 7 fish were incubated in either 1 ml of Cortland medium (control group) or Cortland medium containing 10 μ M Forskolin and 500 μ M IBMX for 4 hours. The incubation media were collected and stored at -20°C until assayed for steroid content.

We have also studied whether there was a cross talk between the cAMP and Ca^{2+} -CaM signalling pathways. In order to compare the effect of W7 and forskolin administration, we have designed an experiment whereby testicular fragments from 6 fish were incubated for 8 hours in Cortland media with either W7 (80 μ M), or 10 μ M Forskolin and 500 μ M IBMX, or with the simultaneous addition of W7+ Forskolin+ IBMX. A control group of testicular fragments incubated in Cortland medium was also included in order to assess the effect of each treatment. All incubations were done for 8 hours, after which the media were collected and stored at -20°C until assayed for steroid content.

5.2.4 Radioimmunoassays (RIA)

Individual samples (50 μ l) were denatured for 1 hour at 80°C and steroid hormones were measured by radioimmunoassay following the methodology described in (Canario and Scott, 1989). Specificity tables for the radioimmunoassays used in this study have been previously published: testosterone (T) and 11-ketotestosterone (11-KT) (Kime and Manning, 1982); 17,20 α -dihydroxy-4-pregnen-3-one (17,20 α -P) (Canario and Scott, 1989); androstenedione (A) (Scott et al., 1984); cortisol (Rotllant et al., 2005) and 11-desoxicortisol (Canario and Scott, 1990).

5.2.5 Thin layer chromatograph (TLC)

Blood plasma and incubation media were used for TLC analysis in order to confirm that the cortisol antibody was specific. Incubation media were extracted by pre-conditioned solid-phase Sep-Pak cartridges (C18, Waters, USA), eluted in ethanol and evaporated under liquid N_2 at 40°C. The extracts were applied and separated for 50 min on TLC plates (Whatman LK6DF silica gel 60A plates, Whatman Inc., New Jersey, USA) with chloroform: methanol (47.5:2.5) as the mobile phase along with [^{3}]cortisol in a parallel lane. Radioactivity was scanned with the radiochromatograph Bioscan (Lablogic, Sheffield, England). Lanes were divided in 0.5 cm fractions, scraped off and steroids eluted with

dichloromethane: ethanol (80:20) and subsequently evaporated under nitrogen. All fractions were subsequently assayed by RIA, using cortisol specific antibody.

5.2.6 Statistics

Steroid and calcium levels are expressed as mean $\pm$ standard error of the mean, and mRNA levels are expressed as the mean ratio of gene of interest by the 18S amplification $\pm$ standard error of the mean. Data was tested for homogeneity of variance and normal distribution and analysed by One-Way ANOVA followed by pairwise multiple comparison procedure (Tuckey test), when appropriate. Groups were considered significantly different for $p < 0.05$ unless otherwise stated and $n = 6-7$.

5.3 Results

5.3.1 The effect of extracellular calcium, CaM inhibition and forskolin on androgen production.

Basal T (Fig. 1a) or 11-KT (Fig. 1b) levels were not affected by either removal of extracellular calcium or by adding EGTA, a calcium chelator, although after 6 hour of incubation, the average levels of testosterone within the group increased 60 % when compared to control levels ($p < 0.2$).

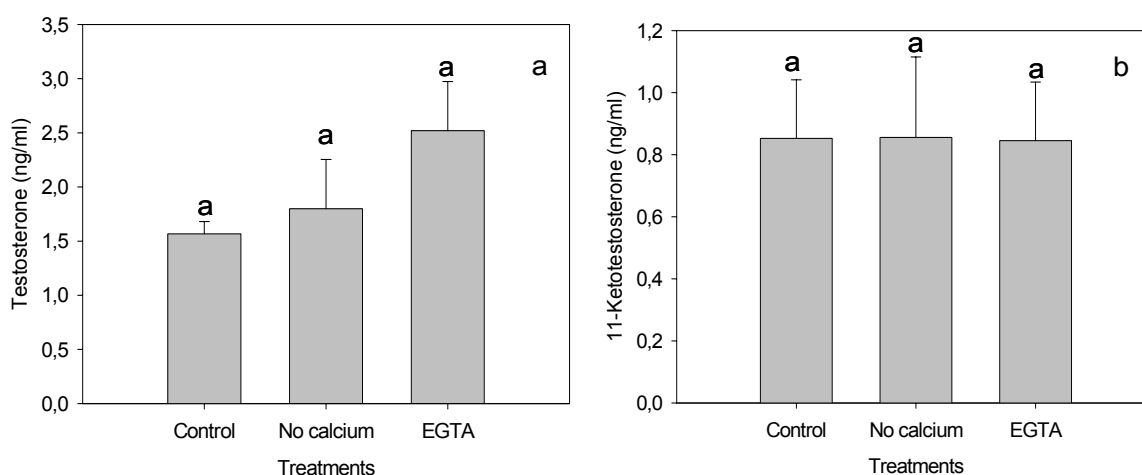


Figure 1- The effect of calcium and EGTA on basal testosterone (a) and 11-ketotestosterone (b) levels in testicular tissue incubations. *In vitro* incubations were performed in Cortland medium for 6 hours at 24°C. Control group consists of testicular tissue incubated in Cortland medium, in the group with no calcium testicular tissue was

incubated in Cortland medium without Ca^{2+} in its composition and in the EGTA group, fragments were incubated in Cortland medium without Ca^{2+} and with the addition of 1 mM EGTA. In all groups $n=7$. Different letters denote statistical differences between the treatment groups when $p<0.05$.

Incubations of testicular tissue with W7 at different concentrations did not affect the basal T levels (Fig. 2a), but the basal 11-KT levels were significantly reduced at 80 μM W7 ($p<0.05$), after 6 hours of incubation (Fig. 2b).

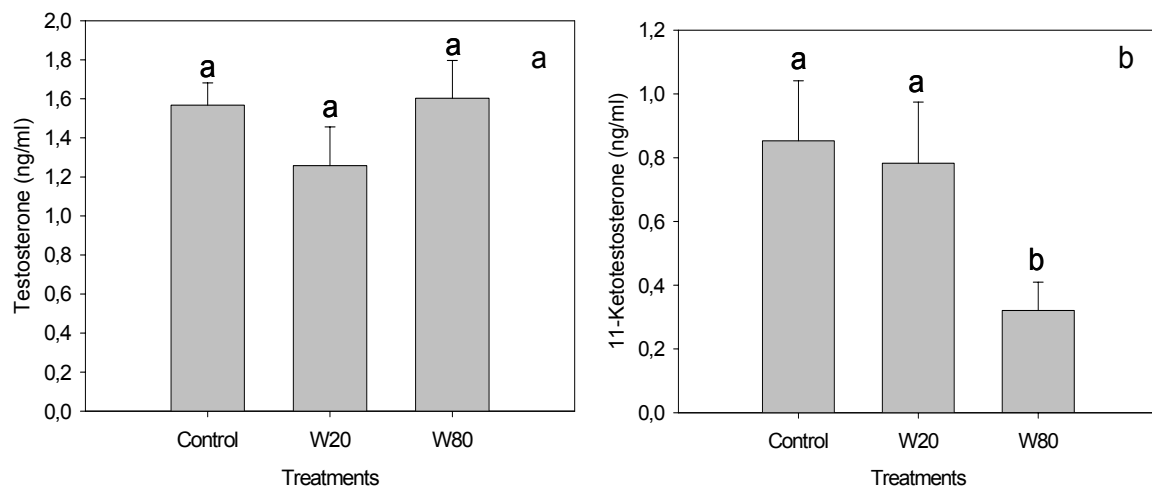


Figure 2- The effect of W7 on basal testosterone and 11-ketotestosterone levels in testicular tissue incubations. *In vitro* incubations were performed in Cortland medium with the addition of 20 μM W7 for 6 hours at 24°C. In all groups $n=7$ and letters denote statistical differences between treatments. Different letters denote statistical differences between the treatment groups when $p<0.05$.

The effect of the adenyl cyclase stimulator, forskolin, was studied in a preliminary experiment that consisted of incubations of 4 hours with or without the stimulator. Thus, addition of forskolin and IMBX to the incubation medium for 4 hours had a significant effect on testosterone production ($p<0.02$), but no effect was detected in 11-KT levels (data not shown).

Considering that the preliminary experiment with forskolin incubations has shown the opposite pattern of W7 incubations, we have hypothesized that there may be a cross-talk between the cAMP and the Ca^{2+} -CaM signalling pathway. In order to test this hypothesis we have designed an experiment where testicular tissue was not only incubated either with W7 or forskolin+ IMBX, but we have also included a treatment group

where the tissues were subjected to simultaneous addition of W7 and forskolin+ IBMX (Fig. 3). However, considering that forskolin had an effect within a shorter time frame than W7, we have decided to perform this experiment for 8 hours to ensure enough time for calmodulin inhibition by W7. Thus, after 8 hours of incubation with forskolin, T and 11-KT production increased significantly ($p<0.004$ and $p<0.003$, respectively), when compared with control levels. Administration of W7 alone reduced the T and 11-KT levels significantly ($p<0.02$ and $p<0.003$, respectively), when compared to control levels. Concomitant administration of forskolin with W7 blocked the forskolin stimulation of T and 11-KT production in the gonads ($p<0.05$).

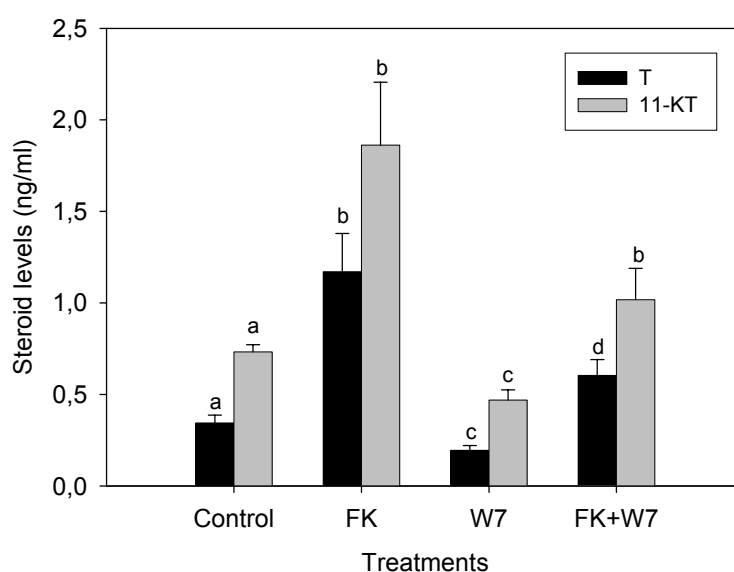


Figure 3- The effect of W7 and Forskolin on basal testosterone (T) and 11-ketotestosterone (11-KT) levels in testicular tissue incubations. *In vitro* incubations were performed in Cortland medium for 8 hours at 24°C. Control group consisted of testicular tissue incubated in Cortland medium. The treatments applied in this experiment consisted of incubations in Cortland medium with the addition of 10 μ M forskolin and 500 μ M IBMX (FK group), or 80 μ M W7 (W7 group), or with the simultaneous addition of 10 μ M FK+ 500 μ M IBMX+ 80 μ M W7 (W7+FK group). In all groups $n=6$. Different letters denote statistical differences between the treatment groups when $p<0.05$.

5.3.2 The effect of W7 and forskolin on cortisol production in testicular tissue.

Administration of W7 to the gonads also resulted in increased cortisol production, cortisol (Fig. 4a) and 11-desoxycortisol (Fig. 4b). Cortisol and 11-desoxycortisol levels were increased significantly ($p<0.03$ and $p<0.02$, respectively) at 80 μ M.

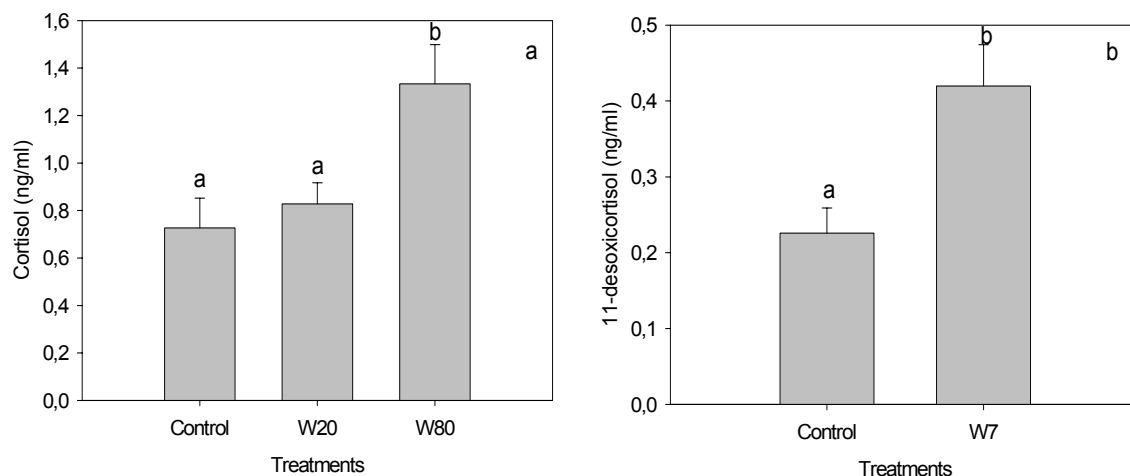


Figure 4- The effect of W7 on basal cortisol levels in testicular tissue incubations. *In vitro* incubations were performed in Cortland medium for 6 hours at 24°C. Testicular tissue was incubated in Cortland medium (Control) or with the addition of 20 μ M or 80 μ M W7, and the cortisol (a) and 11-desoxycortisol (b) levels were measured by RIA with specific antibodies. In all groups $n=7$. Different letters denote statistical differences between the treatment groups when $p<0.05$.

In order to confirm that cortisol RIA were specific, both control and 80 μ M W7 incubation media were separated on TLC and the fractions were analysed by RIA with cortisol specific antibody. Analysis of the fractions showed that control gonads do not produce cortisol, as no cortisol immunoreactivity was detected (Fig. 5a). However, in W7 incubations, the pattern of immunoactivity changed as a large peak was detected within the fraction that correspondes to cortisol (Fig. 5b).

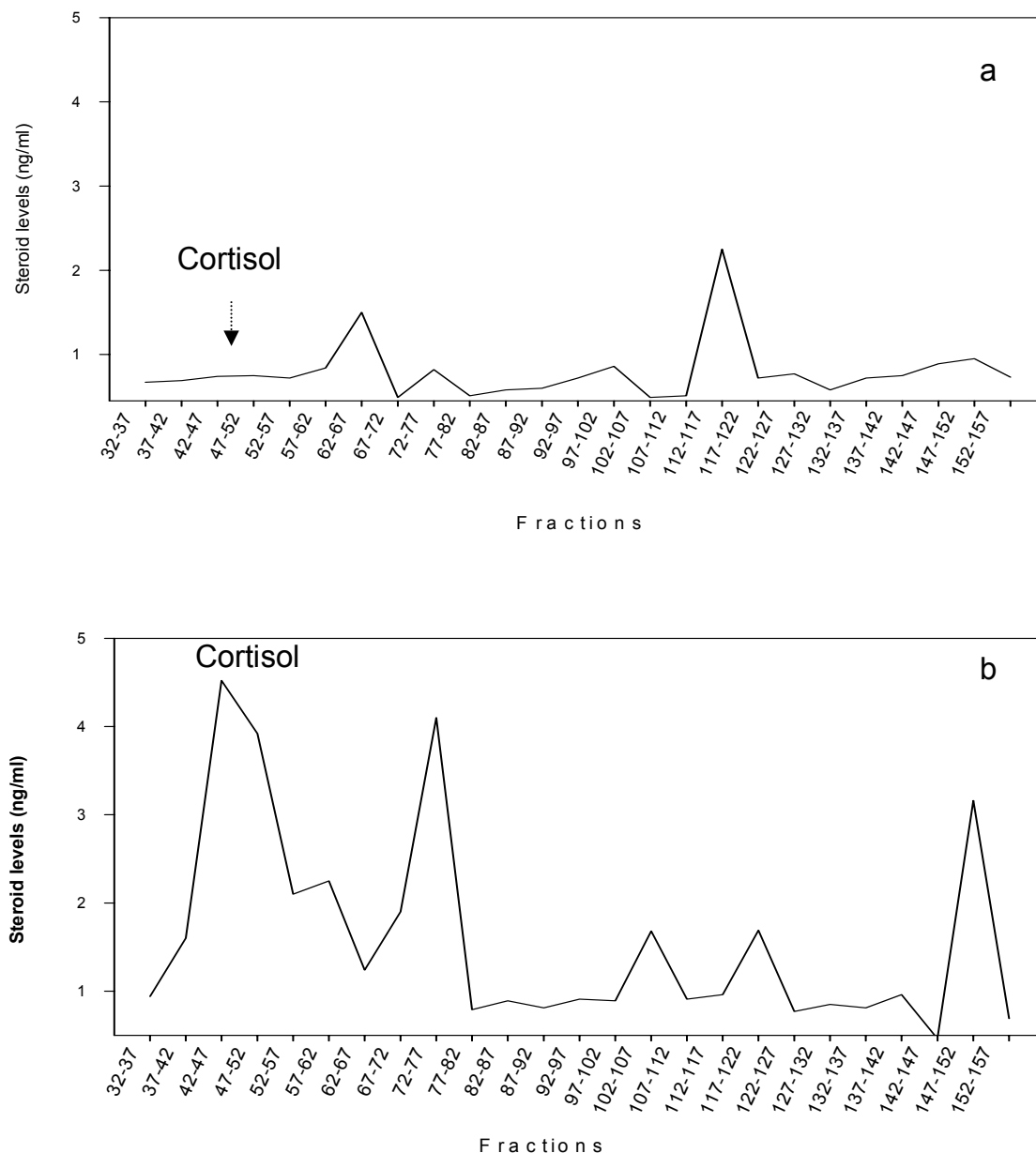


Figure 5- Cortisol levels in control incubation medium (a) and in W7 incubation medium (b) detected in TLC isolated fractions. The extracted steroids were applied and separated for 50 min on TLC plates with chloroform: methanol (47.5:2.5) as the mobile phase along with the cold steroid standards for these steroids, $^{[3]}\text{F}$. Radioactivity was scanned and lanes were divided in 0.5 cm fractions, scraped off and steroids eluted with dichloromethane: ethanol (80:20) and subsequently evaporated under nitrogen. All fractions were analyzed by radioimmunoassay as described in the methods' section.

Similar to what was seen in W7 incubations, treatment with forskolin induced a significant production of cortisol (Fig. 7) ($p < 0.04$).

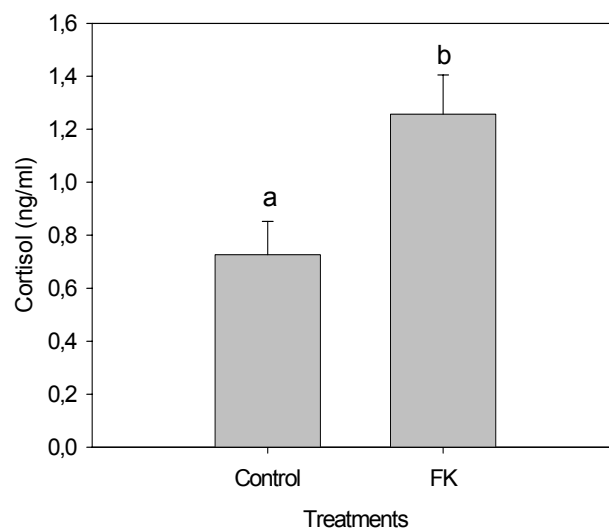


Figure 6- The effect of forskolin on basal cortisol levels in testicular tissue incubations. *In vitro* incubations were performed in Cortland medium for 4 hours at 24°C. Testicular tissue was incubated in Cortland medium (Control) or with the addition of 10 μ M Forskolin and 500 μ M IBMX (FK). In all groups $n=7$. Different letters denote statistical differences between the treatment groups when $p<0.05$.

To further confirm that the inhibition of androgen production by W7 was accompanied by increased cortisol production and not by the increase and/or accumulation of steroids in alternative metabolic routes prior to testosterone, we measured 17, 20 α -dihydroxy-4-pregnen-3-one (17,20 α -P) and androstenedione. W7 did not affect the production of 17,20 α -P (Fig. 7a), but androstenedione production decreased significantly ($p<0.02$) (Fig. 7b).

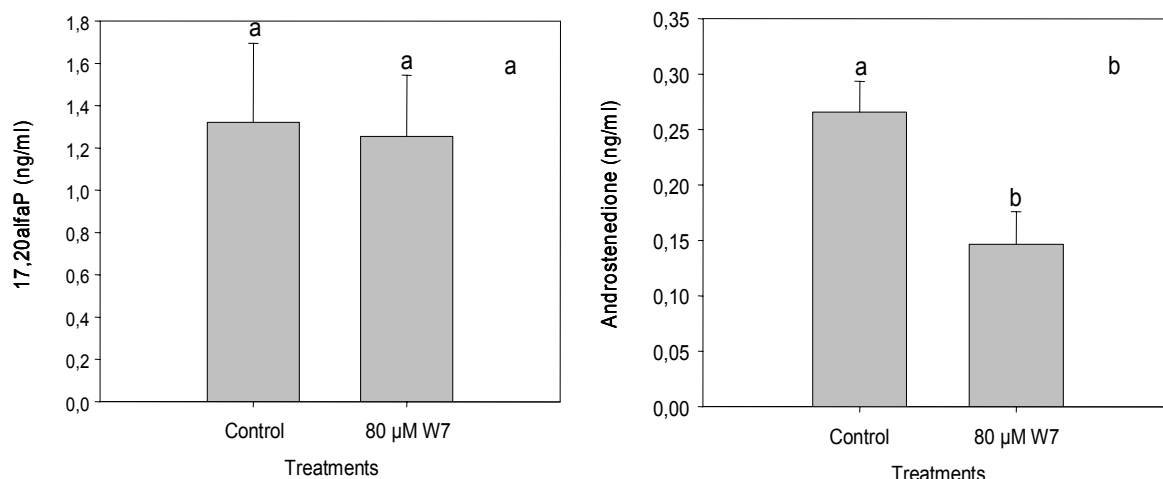


Figure 7- The effect of W7 on basal 17,20α-P and androstenedione levels in testicular tissue incubations. *In vitro* incubations were performed in Cortland medium for 8 hours at 24°C. Testicular tissue was incubated in Cortland medium (Control) or with the addition of 80 μM W7, and the 17, 20 α-Progesterone (a) and androstenedione (b) levels were measured by RIA with specific antibodies. In all groups $n=6$. Different letters denote statistical differences between the treatment groups when $p<0.05$.

5.4 Discussion

In this chapter, we have shown that basal androgen production in testis can be suppressed by blocking available CaM with a CaM inhibitor (W7) and also that steroid production is more sensitive to increased cAMP levels than to altered extracellular Ca^{2+} levels.

In Mozambique tilapia, testosterone basal levels were not affected significantly by calcium removal from the incubation medium or by EGTA treatment, suggesting that extracellular calcium levels alone are not essential for the maintenance of basal testosterone production. This is different from rat testicular tissue where omission of Ca^{2+} from the incubation medium in rat Leydig cell cultures led to reduced LH/hCG stimulated steroid production (Choi and Cooke, 1992; Manna et al., 1999). However, other studies in rat testicular tissue and in the Atlantic croaker ovaries using different extracellular calcium concentrations, no calcium, EGTA (a calcium chelator) or La^{3+} (inhibitor of calcium entry through the plasma membrane) have failed to show a correlation between basal testosterone levels and extracellular calcium levels (Sullivan and Cooke, 1986; Choi and Cooke, 1992; Benninghoff and Thomas, 2005). It is interesting, however, that although the extracellular calcium levels appear to have no effect in basal testosterone levels in the

Atlantic croaker (Benninghoff and Thomas, 2006), administration of calcium ionophores (ionomycin and A23187) or administration of voltage gated calcium channel (VGCC) inhibitors (verapamil and nifedipine) to granulosa cells reduced both hCG stimulated and basal testosterone levels significantly. Contradictory results had been obtained in earlier studies with rat testicular incubations with different calcium channel blockers [verapamil, D600 enantiomers (+) and (-) and cobalt] since all inhibitors blocked LH and dibutyl cAMP stimulated testosterone production but no effect could be seen in basal testosterone production (Moger, 1983). In rat Leydig cells, abolition of the FSH-induced rise of cytosolic calcium by the removal of extracellular calcium and by blockers of both voltage-gated and voltage-independent calcium channels suggested that more than one class of calcium channels is involved in this process (Gorczyńska and Handelsman, 1991). Furthermore, the use of non selective ionophores, like ionomycin, have proven to induce greater effects on cytosolic calcium than ionophore selective for voltage-gated channels (Bay K8644) (Gorczyńska and Handelsman, 1991), suggesting that voltage independent calcium channels were also affected by the ionophore. In fact, exposure of the Sertoli cells to extracellular environment reversing potassium and sodium ion concentrations (high K^+ , low Na^+) causes the elevation of cytosolic calcium, suggesting that depolarizing the Sertoli cell plasma membrane activates the voltage-sensitive calcium channels (Gorczyńska and Handelsman, 1991; Lalevee et al., 1999). Thus, it is possible that the effect of the ionophores used in the Atlantic croaker on basal testosterone production is not due to a selective activation of VGCC but to additive effect due to simultaneous activation of voltage independent calcium channels, which would explain the contradictory results of ionophore administration and calcium withdrawal and/ chelating with EGTA on basal testosterone levels.

Previous studies have demonstrated that gonadotrophin stimulated testosterone production in rat testicular tissue can be blocked by different CaM inhibitors (W7 and trifluoroperazine) (Choi and Cooke, 1992). Likewise, fish ovarian cells incubations with either CaM inhibitor (W7) or CaM inhibitors (KN-63 and KN-93) significantly reduce hCG-stimulated testosterone production (Benninghoff and Thomas, 2005; Benninghoff and Thomas, 2006). Despite the observed reduction of testosterone in hCG stimulated cells, none of the studies detected a significant effect of the inhibitors on basal testosterone levels. However, in the present study testicular tissue of Mozambique tilapia basal testosterone production was inhibited by W7 at the highest concentration (80 μM) during shorter incubation periods than those used with Atlantic croaker ovaries (Benninghoff and Thomas, 2005). These results suggest that either testis is more sensitive to this inhibitor or that the differences are due to different incubation periods or differences between species.

Many studies have focused on the relationship between cAMP and calcium ions in rat Sertoli cells (Gorczyńska et al., 1994; Gorczyńska et al., 1996). Increased cytosolic calcium levels due to FSH in freshly isolated Sertoli cells could be quantitatively reproduced by replicating intracellular cAMP effects through using either a membrane-permeable analogue ((Bu)₂ cAMP) or by direct non receptor-mediated activation of adenylate cyclase (forskolin or cholera toxin) (Gorczyńska and Handelsman, 1991). In rat Sertoli cells, the cAMP-evoked rise in cytosolic calcium involves calcium from both extracellular and intracellular calcium sources (Gorczyńska et al., 1994) a fact corroborated by the significant decrease in cAMP-induced cytosolic calcium rise either in the absence of extracellular calcium or in the presence of verapamil (Grasso and Reichert, 1989). Thus, in rat there is an apparent cross-talk between cAMP signalling and the Ca²⁺ signalling pathways. Incubations of rat testicular tissues (Sullivan and Cooke, 1986; Khanum et al., 1997) and fish ovarian tissues (van der Kraak, 1991; Benninghoff and Thomas, 2006) with forskolin have induced increased accumulation of testosterone levels. Similarly, incubation of tilapia testicular tissue with forskolin for 4 hours increased the testosterone levels significantly, suggesting that the basal testosterone levels are more sensitive to alterations in cAMP levels than to oscillations in CaM levels. Moreover, forskolin stimulation was significantly decreased by the simultaneous administration of the CaM inhibitor, W7, suggesting that the cAMP stimulated testosterone production in tilapia testicular tissue is to some extent dependent on the activation of the CaM signalling pathway. Coincident results have also been reported in studies using CaM inhibitors to block forskolin- or cAMP-induced steroidogenesis in goldfish and Atlantic croaker granulosa cells, providing evidence for a regulatory role of calcium or CaM distal to activation of adenyl cyclase and PKA (van der Kraak, 1991; Benninghoff and Thomas, 2006). Furthermore, administration of inhibitors of CaMKs or VGCC to forskolin stimulated granulosa cells in the Atlantic croaker have also blocked the cAMP stimulated testosterone production (Benninghoff and Thomas, 2006), showing that inhibition of the calcium entry, CaM or CaMK activities will ultimately result in decreased stimulation of testosterone levels by cAMP analogue forskolin. All together these data clearly show that in different fish species, gonadal testosterone production is regulated by two different signalling pathways, via cAMP activation and via CaM activation, and that there is a cross talk between the different signalling pathways for this regulation to be achieved.

17β-Estradiol was not detected in the Mozambique tilapia testicular incubation media and thus it is not likely that the observed reduction in basal testosterone levels in the presence of W7 was due to the increased aromatization of testosterone. An alternative possibility would be increased conversion to 11-KT. However, the levels of 11-KT in fish treated with W7 were significantly decreased at 80 μM after 6 hours of incubation when no

effect could be seen in testosterone levels, suggesting that the enzymatic steps for testosterone production are less sensitive to calmodulin inhibition than the subsequent steps for its conversion into 11-KT. Thus, the enzymes involved in the conversion of T into 11-KT, 11 β -hydroxylase (11 β -OH) and/or 11 β -hydroxysteroid dehydrogenase (11 β -HSD) might be potential targets for regulation by CaM. Nonetheless, as with testosterone, calcium remobilization or addition of EGTA had no effect on the 11-KT levels in Mozambique tilapia.

Interestingly, and in contrast to what we had seen with CaM inhibition, 11-KT production seemed to be less sensitive to cAMP regulation when compared to T, since we only detected significant differences after 8 hours of incubation with the cAMP signalling activator forskolin (4 hours for T). This suggests the increased 11-KT levels are more likely due to the forskolin stimulated increase of T levels which is then converted to 11-KT. Nonetheless, concomitant administration of W7 with forskolin for 8 hours strongly decreased basal T and 11-KT production, showing that there is a cross-talk between both signalling pathways in the regulation of T production and conversion into 11-KT. Altogether these results suggest that modification of either cAMP or CaM levels can alter basal T and 11-KT production.

To determine whether the inhibition of T and 11-KT production by W7 could be due to targeting the steroidogenic pathway at steps prior to androgen production that could be shifting steroid production to C21 steroids we measured 17,20 α -P, one of the main steroids pregnenes produced by male Mozambique tilapia (Oliveira et al., 1996; Rocha and Reis-Henriques, 1996), but found no effect of W7. This suggests that the decreased androgen levels are not due the accumulation of progesterone metabolites in earlier steps of steroidogenesis.

Previous studies have shown that the testis Mozambique tilapia is able to synthesize 21-hydroxylated steroids including desoxycortisol and its metabolites (Kime and Heyder, 1983; Rocha and Reis-Henriques, 1996). The TLC analysis of incubates from control testis did not detect any cortisol in testicular tissue, although residual 11-desoxicortisol was detected. These results suggest that in control conditions, although 11-desoxicortisol is being produced at residual levels, the glucocorticoid pathway is suppressed in the gonads, and that the cortisol immunoreactivity derived from cross-reaction with other, possibly 21-hydroxylated steroids under the control of 21-hydroxylase (CYP21). In W7-treated testis, TLC analysis detected an increase in 11-desoxicortisol production and, surprisingly, significant amounts of cortisol. These results suggest that CaM inhibition favoured glucocorticoid synthesis, possibly through the inhibition of 17-hydroxylase/C17-20 lyase (CYP17), in detriment of androgen production. They also suggest that 11 β -OH is not a target for CaM and that the stronger inhibition observed for

11-KT production compared to T is largely due to blockage of 11 β -HSD mRNA expression or enzyme activity. There are several possible regulatory steps being affected by the calmodulin inhibitor within the steroidogenic pathway.

There are three enzymes within the pathway for the conversion of androstenedione to testosterone and 11-KT: 17 β -HSD, 11 β -OH and 11 β -HSD. Earlier studies have shown that glucose is required for optimal hCG-stimulated testosterone production in rat Leydig cells (Muroso et al., 1982) and that the activity of 17 β -HSD is under ATP control (Khanum et al., 1997). Addition of W7 to the incubation medium significantly decreases 17 β -HSD activity and it has an additive effect when administered with an ATP blocker (KT-5720) (Khanum et al., 1997). The activity of the rat testicular 11 β -HSD1 has been shown to be regulated by calcium and CaM inhibitors but the effect the CaM inhibitors had no detectable effects on testosterone production (Ge and Hardy, 2002). Moreover, 21-hydroxylase, responsible for the initial steps in cortisol production (the conversion of 17 α -hydroxyprogesterone into 11-desoxicortisol) and the 17-hydroxylase, responsible for the conversion of progesterone to 17-hydroxyprogesterone and androstenedione, are both regulated by extracellular calcium in the adrenals of rats, suggesting that cortisol and androgen production is target for calcium regulation in steroidogenic tissues (Bird et al., 1995; Bird et al., 1998). Altogether these studies suggest that cortisol and androgen production in steroidogenic tissues is regulated by the Ca²⁺ signalling pathway. Thus, it appears that the CaM inhibitor W7 is affecting multiple steps within the steroidogenic pathway in Mozambique tilapia testis that is ultimately leading to a shift in steroid production favouring glucocorticoid production in detriment of androgen. This observation also raises the possibility of a mechanism for suppression of glucocorticoid production in the testis, perhaps to avoid adverse effects of excessive glucocorticoids on reproductive function.

In a few studies in fish an interaction between 11-KT and glucocorticoids biosynthesis has been observed and it has been suggested that it has implications in reproductive physiology and behaviour in relation to sex change (Perry and Grober, 2003). For example, administration of cortisol to common carp, *Cyprinus carpio*, increased the accumulation of the 11-KT precursor 11 β -hydroxyandrostenedione, which after cortisol clearance was converted to 11-KT and suggesting possible competitive inhibition induced by high cortisol levels (Consten et al., 2001). Moreover, a direct connection between the reproductive and stress axes have been clearly defined in teleosts which share the same enzymes, 11 β -OH and 11 β -HSD for the metabolism of androgens and glucocorticoids. This relationship is detectable in the gonad of a sex changing grouper (*Epinephelus coioides*) before sex inversion from male to female, when the gonads are able to produce both androgens steroids and glucocorticoids, but not after sex inversion (Lee et al., 2000).

In summary, testosterone and 11-ketotestosterone production in Mozambique tilapia testis is regulated by CaM and cAMP-PKA signalling pathways, with a significant cross-talk between both pathways. Furthermore, blocking the CaM signalling pathway affects the net direction of steroid production as the decrease in androgen production shifted the pathway towards favouring the activation of glucocorticoid production (11-desoxicortisol and cortisol). These results suggest that there may be substrate competition by the enzymes that regulates the direction of steroid synthesis in the testis and that the activation of the CaM signalling pathway is a required step for determining the final outcome of steroidogenesis in the testis.

Chapter VI

Calmodulin inhibitor W7 blocks testis steroid production and increases *DAX1* and *DAX2* mRNA levels in adult male Mozambique Tilapia (*Oreochromis mossambicus*) *in vivo*.

With: Juan Fuentes, Olinda Almeida, Deborah M Power and Adelino VM Canário.
Manuscript in preparation.

Abstract

The role of the calcium-binding protein calmodulin (CaM) in the regulation of androgen production in testis of Mozambique tilapia (*Oreochromis mossambicus*) *in vitro* has already been shown in chapter 5. The objective of this study was to determine whether CaM regulates testicular androgen production, *in vivo*, in male Mozambique tilapia and to identify the genes that may be involved in this regulation. Males were injected with 140 μ M of the CaM inhibitor W7 or with the vehicle only (Control group) and 24 hours later were sacrificed and testis and blood samples collected. Calcium and steroid levels in blood and the mRNA expression levels of *DAX1*, *DAX2*, estrogen receptors (α and β), androgen receptor (*AR* β), *CYP11B1*, *CYP19*, *CALM*, *SOX9.1* and *SOX9.2* genes and *SF1* were measured. W7 administration decreased total calcium (18%), testosterone (88%) and 11-ketotestosterone (90%) levels in plasma. In the gonads, fish treated with W7 had significant upregulation of *DAX1*, *DAX2* and *AR* β mRNA levels and decreased *ER* α mRNA levels. The expression levels of *SF1*, *SOX9.1*, *SOX9.2*, *CYP19*, *CYP11B1* and *CALM* were not affected by W7. Moreover, administration of W7 increased the *DAX1/SF1* mRNA ratio in testis. These results suggest that modulation of CaM levels within the cell modifies *DAX1* transcriptional levels, supporting the hypothesis of common gene networks of CaM signalling and *DAX1* in steroid production in fish.

6.1 Introduction

Different *in vivo* and *in vitro* studies have shown that androgens play a crucial role in the development, maintenance, and regulation of male phenotype and reproductive physiology in fish. In this respect, androgen treatments have been widely used to control the sex phenotype in different fish species (Baroiller et al., 1999). Even though testosterone (T) is the main mammal androgen, 11-Ketotestosterone (11-KT) (and to a lesser extent T) is considered the main androgen of the teleost testis (Borg, 1994; Devlin and Nagahama, 2002). In addition, androgens are also important stimulators for teleost reproductive behavior (Bass, 1992) and secondary sexual characters (Borg, 1994). In the Tilapia (*Oreochromis mossambicus*), social interactions modulate the androgen circulating levels, so that male individuals have short term increased androgen in response to courtship interactions, during the establishment of dominance amongst males and throughout the establishment of the social status within a group of male fish (Oliveira et al., 1996; Borges et al., 1998). Similarly, in another cichlid species, *Astatotilapia (Haplochromis) burtoni*, social status regulates male fertility, so that dominant males contain higher levels of 11-ketotestosterone and testosterone (Parikh et al., 2006). The higher levels of circulating androgens in dominant males have behavioural and physiological consequences, and in tilapia (*Oreochromis mossambicus*), it has been proposed that T is involved in keeping the fish in a state of behavioural readiness allowing a rapid shift to a more aggressive behavior if favourable opportunities are presented, while 11KT is more directly involved in gamete development and maintenance of male secondary sexual characteristics (Oliveira et al., 1996). Thus, the study of any regulatory mechanism controlling sex steroid production in male Tilapia would be of particular importance in understanding the reproductive and behavioural responses in this species.

Both *in vivo* and *in vitro* studies on different teleost species have shown that spermatogenesis is regulated by androgens directly on the gonad and through positive feedback mechanisms in the brain and pituitary (Montero et al., 1995; Cavaco et al., 2001). Androgen regulatory actions are mediated through the androgen receptor (AR). In fact, in male mice, mutations in the AR can lead to several abnormalities ranging from complete sex reversal to infertility (Brown, 1995). Interestingly, estrogen and its receptor *ER* α , but not *ER* β , have also been shown to be important for the maintenance of male fertility. Although disruption of *ER* β in male mice does not impair the reproductive function (Krege et al., 1998), *ER* α null male mice present reduced fertility and dramatically decrease in epididymal sperm counts, suggesting a primary role in regulating spermatogenesis (Ogawa et al., 1997).

Apart from the steroidogenic control of fertility, mutations or ablation of different transcription factors involved in gonadal differentiation and maturation have also been shown to result in impaired steroid production and/or infertility problems in male gonads. *DAX1*, *SF1* and *SOX9* are transcription factors that are involved in male gonadal sex determination. Mutations in human *DAX1* cause X-linked adrenal hypoplasia congenita (Zanaria et al., 1994) and males develop primary adrenal failure in infancy or childhood. Furthermore, targeted disruption of *DAX1* causes disordered spermatogenesis and infertility in mice (Yu et al., 1998b). *SF1* is a transcription factor that regulates the transcription of an array of genes involved in male sexual differentiation, Müllerian regression, testicular descent, steroidogenesis, and reproduction (Parker and Schimmer, 1997). Homozygous deletion of *Sf1* (-/-) in mice results in complete gonadal and adrenal agenesis and phenotypic XY sex reversal (Luo et al., 1994). Mutations in the transcription factor *SOX9* are responsible for the campomelic dysplasia, a rare disease in which approximately 75% of the male patients present gonadal dysgenesis leading to sex reversal (Lynch et al., 1993; Hageman et al., 1998). Thus, mutations in any of these transcription factors compromise both male gonadal development and fertility.

Altogether, these studies show that in order to properly develop and maintain a functional testis, the appropriate levels of steroid enzymes and receptors, as well as of different transcription factors are necessary. However, we still lack data on which factors regulate the levels of these genes within the gonads, and also lack data on their function and regulation in adult gonads. In chapter 5 we showed that steroid production in Mozambique tilapia testis was modulated by calmodulin (CaM). The objective of this chapter was to test if *in vivo* administration of W7 affected the plasma steroid levels. In order to further characterize the impact of CaM inhibition on the testis transcriptional activity we have also analysed gene expression of *DAX1*, as hypothesized in the previous chapters, as well as the gene expression of steroid enzymes (*CYP19* and *CYP11B1*), steroid receptors (*AR α* and *ER α* and *ER β*), transcription factors (*DAX2*, *SOX9* genes and *SF1*) and *CALM*.

6.2 Methods

6.2.1 Fish

Mature Mozambique tilapia (*Oreochromis mossambicus*) (average body mass 110 g) were obtained from a stock raised at the University of Algarve and maintained in freshwater under natural conditions of water temperature and photoperiod.

6.2.2 Injections with calmodulin inhibitor W7

Mature male tilapias were randomly distributed between two freshwater tanks (n=7 each) and acclimated for 2 weeks prior to the start of the experiment. At the start of the experiment fish were anaesthetized with 2-phenoxyethanol (1:10,000 2-phenoxyethanol: seawater; Sigma-Aldrich, Madrid, Spain), and all fish were weighted. Fish from one of the tanks received an intraperitoneal injection of 10% DMSO in saline solution (150 mM NaCl) (1 µl/g body weight) containing W7 (N-(6-aminohexyl)-5-chloro-1-naphtalenesulfonamide) (50 µg/g body weight; Sigma-Aldrich, Madrid, Spain) and fish from the other tank received an injection of 10% DMSO in saline solution (150 mM NaCl) (vehicle control). After 24 hours, fish were anaesthetized and blood samples collected by caudal vessel puncture into heparinised (ammonium heparin, 30 units/ml; Sigma-Aldrich) 2 ml syringes. Blood samples were centrifuged for 5 min (at 10,000 r.p.m.), and the plasma transferred to 1.5 ml vials, immediately frozen in liquid N₂ and stored at -20°C. After collection of blood samples, all fish were killed by decapitation and testicular tissue removed, frozen in liquid N₂ and stored at -80°C for subsequent analysis.

6.2.3 Calcium measurements

Individual total plasma calcium (bound and free) were measured in triplicate for each blood sample. Total plasma calcium was measured by colorimetric assay (Sigma-Aldrich procedure no. 587), according to the manufacturer's instructions.

6.2.4 Radioimmunoassays (RIA)

Individual plasma samples (50 µl) were denatured for 1 hour at 80°C and steroid hormones were measured by radioimmunoassay following the methodology described in (Canario and Scott, 1989). Specificity tables for the radioimmunoassays used in this study have been previously published: testosterone (T) and 11-ketotestosterone (11-KT) (Kime and Manning, 1982); estradiol-17β (Guerreiro et al., 2002); cortisol (Rotllant et al., 2005) and 11-desoxicortisol (Canario and Scott, 1990).

6.2.5 RNA isolation and Reverse transcription

Total RNA was isolated with TRI reagent (Sigma-Aldrich, Madrid, Spain) according to the manufacturer's instructions. Total RNA was treated with the dDNA- free Kit (Ambion, California, USA) and 1 µg RNA was reverse-transcribed in 20 µl at 37°C for 60 min., using

40 U of MMLV-RT, 1 mM dNTPs, 6 U of RNAGuard RNase inhibitor (Amersham Biosciences) and 1 µg random-hexamers (pd (N) 6, Amersham Biosciences) and subsequently heated at 65°C for 10 min to inactivate the enzyme and stored at -20°C.

6.2.6 Real-Time PCR

Real-Time PCR analysis was performed using the qPCR MasterMix Plus for SYBR® Green I (Eurogentec) and the Icyler iQ™ Real-Time PCR Detection System (BioRad) (see chapter 3 for detailed information). Primers for the different genes analyzed were designed based on available sequences in Genebank database (for primer sequences and conditions see table 1).

Table 2- Primers and conditions for gene expression analysis.

<i>Primers</i>	<i>Sequence</i>	<i>Temperature</i>	<i>Sequence Accession number</i>
DAX1tiIRT_Fw DAX1tiIRT_Rv	GCTCTAAACGAGCACGTCAGG CACTGGCGGGCTGATGGCCCTCAG	68°C	DQ269443
DAX2tiIRT_Fw DAX2tiIRT_Rv	ATGAGCACGTGGGGACGGTGTA CAGTCCC GCCACCGCCTCCGTG	66°C	DQ269442
SF1tiIRT_Fw SF1tiIRT_Rv	GCTGGTCTGAACTTCTGGTCTTGG GACAGCTGGACCTCCTGGCCG	64°C	BAC75890
Cyp19tiIRT_Fw Cyp19tiIRT_Rv	CACTGGCCTCATGCACAAAACCG GGCTGGAAGTAACGATTGGGTACC	65°C	AF472621
Erbeta2tiIRT_Fw Erbeta2tiIRT_Rv	GGCTCTGACACTTCCTCTGAACAG TGTCAGCAGCTGTGGCGGTCTGG	68°C	DQ462608
EralfatiIRT_Fw EralfatiIRT_Rv	TGGGCGATGGATGCGGTGAGC CCTGCAGAGCTGTGCAGCGG	62°C	Q9YH33
ArbetatiIRT_Fw ArbetatiIRT_Rv	GTCACCTACAACGACACCAGGT GTCCGAGCAGATGAGGCA	60°C	BAB20082
CalmodtiIRT_Fw CalmodtiIRT_Rv	GATACAGACAGTGAGGAGGAG GTCATAACGTACCGTAGCTCTG	62°C	Q6R520
Cyp11B1bassRT_Fw Cyp11B1bassRT_Rv	CGGAGGGGTTGACACGACAG CCTCACCTGCTGCCTCACCATC	62°C	AAQ04665
Sox9.1bassRT_Fw Sox9.1bassRT_Rv	CAGCAGGGACAGCAGCAACAGCA CAGCAGCTCTGTCTTGATCTGGGTC	65°C	See chapter 3
Sox9.2bassRT_Fw Sox9.2bassRT_Rv	GCCTGGATGTCCAAGCAGC GCTCTGTCTTAATCTGGGTGGT	62°C	See chapter 3
18SF1RT_Fw 18SR1RT_Rv	CGATCAGATACCGTCGTAGTTC CCCTCCGTCAATTCCTTTA	60°C	See chapter 3

The PCR conditions for the reactions were: 95°C for 10 minutes, followed by 45 successive cycles of 95°C for 10 sec for denaturing, 61-68°C for 10 sec. for annealing and 10 sec. for extension of amplified products. The PCR were performed in 96-well micro

plates (Biorad). All measures were performed in duplicates with a variation of less than 5% between replicates. For detailed procedure see chapter 3.

6.2.7 Statistics

Steroid and calcium levels are expressed as mean $\pm$ standard error of the mean, and mRNA levels are expressed as the mean ratio of gene of interest by the 18S amplification $\pm$ standard error of the mean. Data was tested for homogeneity of variance and normal distribution and analysed as appropriate by One-Way ANOVA followed by pairwise multiple comparison procedure (Tuckey test). Groups were considered significantly different for $p < 0.05$ unless otherwise stated. $n=6$, unless otherwise stated.

6.3 Results

6.3.1 The effect of W7 on total Calcium levels in plasma

Fish treated with W7 had significantly lower plasma Ca^{2+} levels than those from control fish ($p < 0.005$) (Fig. 1).

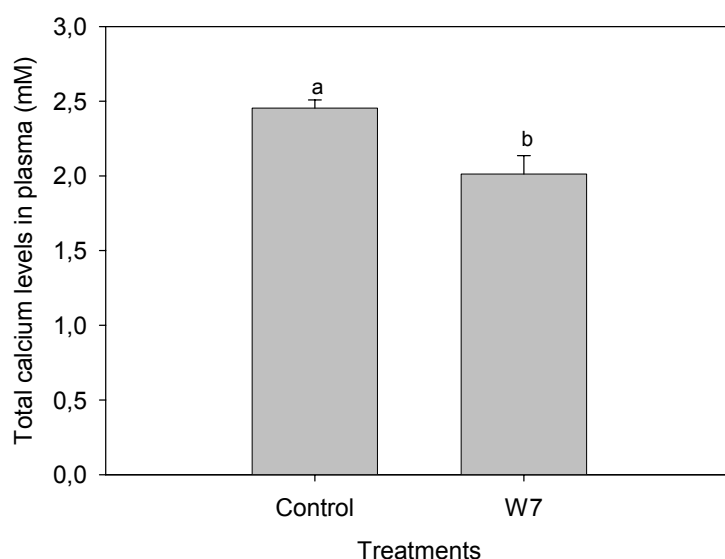


Figure 1- The effect of W7 on total Ca^{2+} levels circulating in plasma. Control group were injected with 10% DMSO in saline solution (150 mM NaCl) and W7 injected group were injected with W7 (50 $\mu\text{g/g}$ body weight) dissolved in 10% DMSO/150 mM NaCl. Both groups consist of $n=7$. Data are expressed as mean $\pm$ standard error of the mean. Different letters denote statistical differences between the treatment groups when $p < 0.05$.

6.3.2 The effect of W7 on sex steroid levels in plasma

The levels of plasma T and 11-KT (Fig. 2) were significantly decreased ($p < 0.001$) 24 hours after W7 injection (reduction of 88 and 90%, respectively).

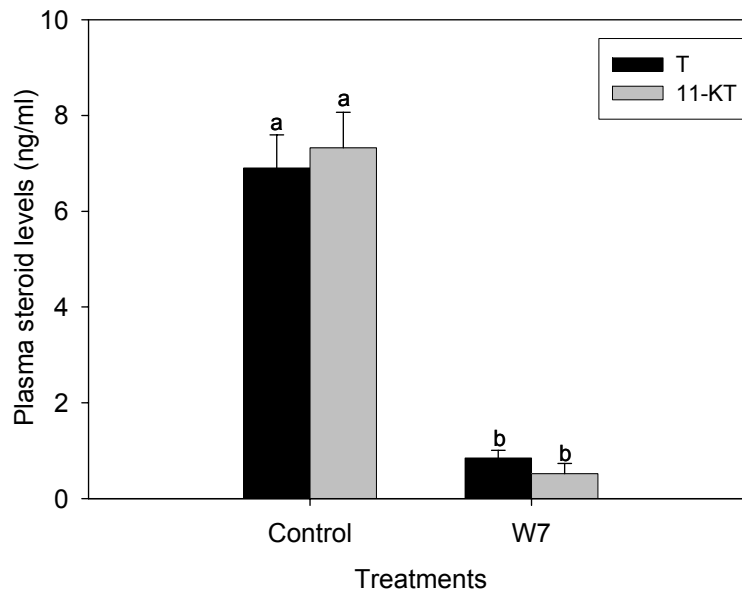


Figure 2- The effect of W7 on plasma testosterone (T) and 11-Ketotestosterone (11-KT) levels of male Tilapia. Control group were injected with 10% DMSO in saline solution (150 mM NaCl) and the treatment group (W7 injected) were injected with 140 μ M W7, dissolved in 10% DMSO/150 mM NaCl. In each treatment group $n=7$. Different letters denote statistical differences between the treatment groups when $p < 0.05$.

In contrast, plasma cortisol levels (Fig. 3) were not significantly different between treatment groups ($p=0.075$), although increased by 38% when compared to control levels.

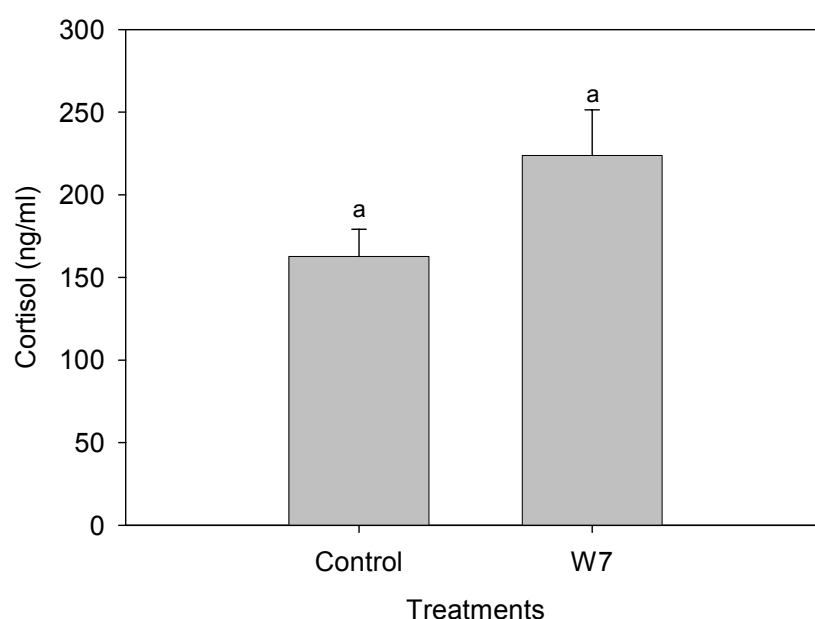


Figure 3- The effect of W7 on plasma cortisol levels of male Tilapia. Control group were injected with 10% DMSO in saline solution (150 mM NaCl) and the treatment group (W7 injected) were injected with 140 μ M W7, dissolved in 10% DMSO/150 mM NaCl. Letters denote significant differences between treatments. In all treatment groups $n=7$. Different letters denote statistical differences between the treatment groups when $p<0.05$.

6.3.3 The effect of W7 injection on calmodulin mRNA levels in testis.

Administration of W7 did not influence calmodulin mRNA levels in testis (Fig. 4).

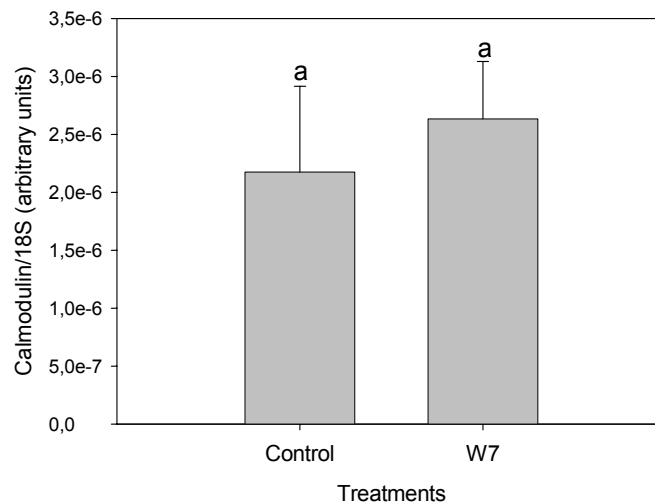


Figure 4- The effect of W7 on calmodulin mRNA levels in tilapia testis. Control group was injected with 10% DMSO in saline solution (150 mM NaCl) and the treatment group (W7) was injected with 140 μ M W7, dissolved in 10% DMSO/150 mM NaCl. 24 hours after the injection, the fish were killed and the gonads were removed. mRNA was isolated, reverse transcribed to cDNA and used for real time PCR with calmodulin specific primers (for primer sequences see the method section). In each group $n=6$. Different letters denote statistical differences between the treatment groups when $p<0.05$.

6.3.4 The effect of W7 on *CYP11B1* and *AR β* mRNA levels in testis

The mRNA level of *CYP11B1* decreased 24 hour after injection of W7 (Fig. 5a). In contrast, mRNA levels of *AR β* (Fig. 5b) were significantly increased by W7 treatment ($p<0.02$).

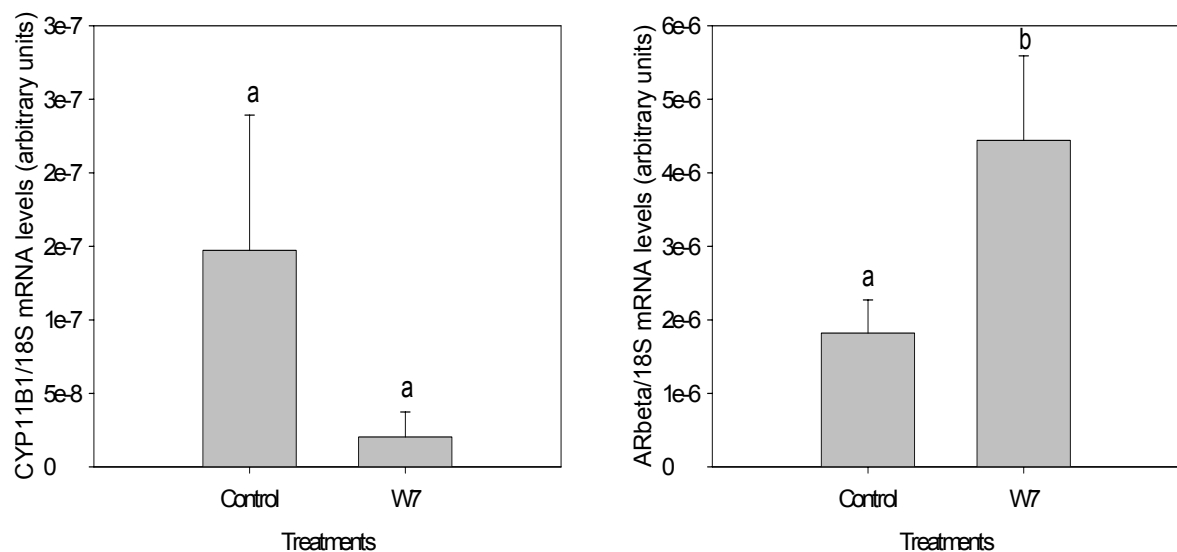


Figure 5- The effect of W7 on *CYP11B1* (a) and *ARβ* (b) mRNA levels in tilapia testis. Control group was injected with 10% DMSO in saline solution (150mM NaCl) and the treatment group (W7) was injected with 140 μM W7, dissolved in 10% DMSO/150 mM NaCl. 24 hours after the injection, the fish were killed and the gonads were removed. mRNA was isolated, reverse transcribed to cDNA and used for real time PCR with *CYP11B1* and *ARβ* specific primers (for primer sequences see the method section). Letters denote statistical differences between treatment groups. In each group $n=6$. Different letters denote statistical differences between the treatment groups when $p<0.05$.

6.3.5 The effect of W7 on mRNA levels of *CYP19* and estrogen receptors in testis

The mRNA level of *CYP19* (Fig. 6a) and *ERβ2* (Fig. 6b) were not affected by W7. In contrast, the mRNA levels of *ERα* (Fig. 6c) were significantly decreased by W7 treatment ($p<0.05$).

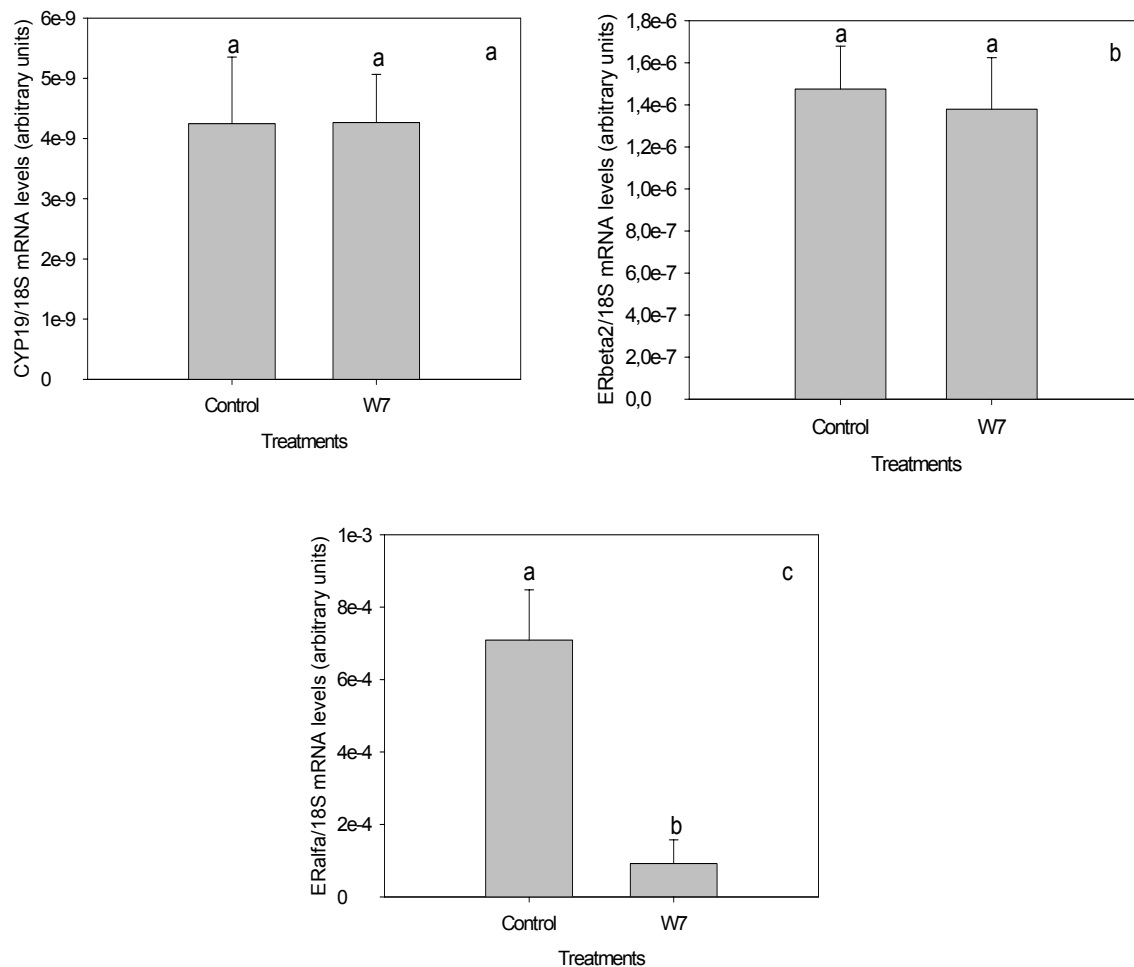


Figure 6- The effect of W7 on *CYP19* (a), *ERβ2* (b) and *ERα* (c) mRNA levels in tilapia testis. Control group was injected with 10% DMSO in saline solution (150mM NaCl) and the treatment group (W7) was injected with 140 μ M W7, dissolved in 10% DMSO/150 mM NaCl. 24 hours after the injection, the fish were killed and the gonads were removed. mRNA was isolated, reverse transcribed to cDNA and used for real time PCR with *CYP19*, *ER α* and *ER β2* specific primers (for primer sequences see the method section). Letters denote statistical differences between treatment groups. In each group $n=6$. Different letters denote statistical differences between the treatment groups when $p<0.05$.

6.3.6 The effect of W7 on *DAX1*, *DAX2* and *SF1* mRNA levels in testis

The mRNA levels of *DAX1* (Fig. 7a) and *DAX2* (Fig. 7b), were significantly increased ($p<0.02$ and $p<0.03$, respectively) 24 hours after injection with W7. In contrast, the *SF1* mRNA levels (Fig. 7c) were not affected by treatment.

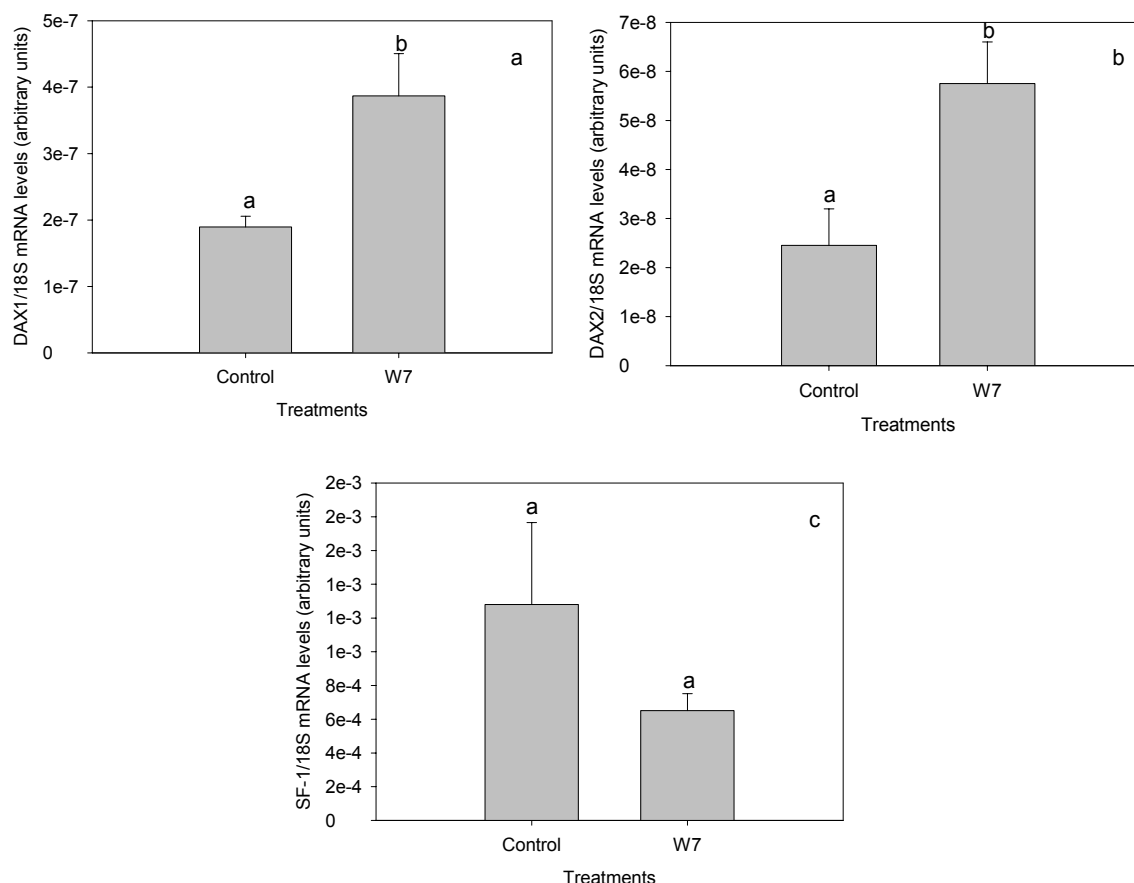


Figure 7- The effect of W7 on *DAX1* (a), *DAX2* (b) and *SF1* (c) mRNA levels in tilapia testis. The control group was injected with 10% DMSO in saline solution (150 mM NaCl) and the treatment group (W7) was injected with 140 μ M W7, dissolved in 10% DMSO/150 mM NaCl. 24 hours after the injection, the fish were killed and the gonads were removed. mRNA was isolated, reverse transcribed to cDNA and used for real time PCR with *DAX1*, *DAX2* and *SF1* specific primers (for primer sequences see the method section). In each group $n=6$. Different letters denote statistical differences between the treatment groups when $p<0.05$.

Interestingly, the *DAX1/SF1* ratio (Fig. 8) was significantly increased 24 hours after administration of W7 ($p<0.01$).

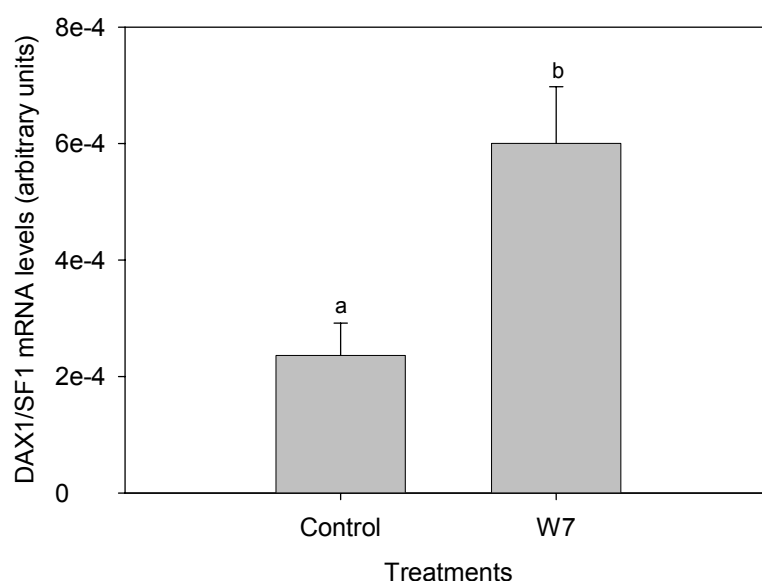


Figure 8- The effect of W7 on DAX1/SF1 ratio in tilapia testis. The control group was injected with 10% DMSO in saline solution (150 mM NaCl) and the treatment group (W7) was injected with 140 μ M W7, dissolved in 10% DMSO/150 mM NaCl. 24 hours after the injection, the fish were killed and the gonads were removed. mRNA was isolated, reverse transcribed to cDNA and used for real time PCR with *DAX1* and *SF1* specific primers (for primer sequences see the method section). In each group $n=6$. Different letters denote statistical differences between the treatment groups when $p<0.05$.

6.3.7 The effect of W7 on SOX9.1 and SOX9.2 mRNA levels in testis

The mRNA levels of SOX9.1 (Fig. 9a) and SOX9.2 (Fig. 9b) did not change significantly after W7 treatment.

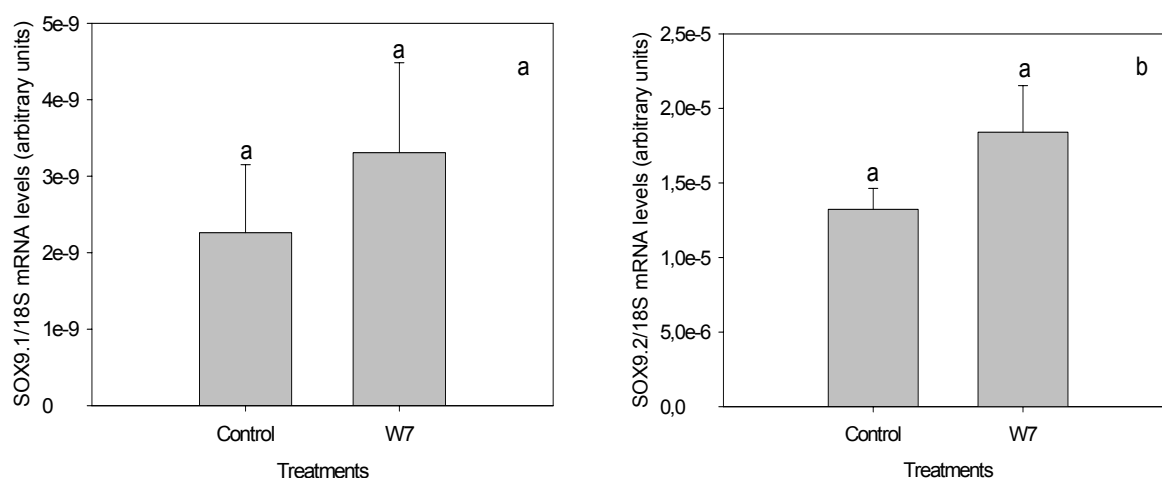


Figure 9- The effect of W7 on SOX9.1 (a) and SOX9.2 (b) mRNA levels in tilapia testis. Control group was injected with 10% DMSO in saline solution (150 mM NaCl) and the treatment group (W7) was injected with 140µM W7, dissolved in 10% DMSO/150 mM NaCl. 24 hours after the injection, the fish were killed and the gonads were removed. mRNA was isolated, reverse transcribed to cDNA and used for real time PCR with SOX9.1 and SOX9.2 specific primers (for primer sequences see the method section). In each group $n=6$. Different letters denote statistical differences between the treatment groups when $p<0.05$.

6.4 Discussion

To our knowledge, this is the first study to date demonstrating an effect of a CaM inhibitor on sex steroid production in an *in vivo* model. In previous studies only CaM kinase inhibitors have been tested *in vivo* in hamsters, mainly for their effectiveness on light-induced signalling phase shifts and c-fos expression in the hamster SCN (Yokota et al., 2001). Studies with the W7 have shown that the subcellular site of action of this inhibitor is similar to that of CaM and that this agent penetrates the cell membrane (Hidaka et al., 1981), suggesting that it probably interacts with CaM mostly in the cytoplasm.

CaM typically acts as a molecular switch that responds to elevated free calcium levels. However, in this study, the plasma calcium levels decreased 18% in fish injected with the CaM inhibitor. Nonetheless, we did not measure the intracellular calcium level in gonad cells and we do not know whether the actual intracellular calcium levels were affected by the inhibitor.

CaM inhibition of steroid production by the tilapia testis is in agreement with the results that we have obtained *in vitro* (chapter 5). Nonetheless, considering the severe impact on androgen production obtained in the present study, and considering that the inhibitor was administered systemically, it is likely that W7 is blocking CaM also in extra gonadal sites, mainly the brain-pituitary axis. The role of brain and pituitary in the regulation of reproduction and steroidogenesis in vertebrates is well known, namely the regulatory actions of gonadotrophin-releasing hormone (GnRH) on gonadotrophin (GtH: FSH and LH) release from the pituitary has decisive actions on the synthesis of androgen production (Walker and Cheng, 2005). In rat pituitary cells, CaM disruption by W7 and over expression of a Ca^{2+} -binding-deficient CaM mutant resulted in blockade of GnRH-induced ERK (but not JNK) signalling (Roberson et al., 2005). In addition, it has also been demonstrated in pituitary cells that GnRH stimulates Ca^{2+} -CaMK II phosphorylation within gonadotropes, which is determinant for signal transduction of the α - subunit and LH β transcriptional responses to GnRH. Furthermore, GnRH stimulation was completely blocked by the specific Ca/CaMK II inhibitor, KN-93 (Haisenleder et al., 2003). These results suggest that blocking the CaM signalling pathway *in vivo* will most likely block GnRH and GtH production in the hypothalamus and pituitary and thus, block stimulation of androgen production in the gonads. It is therefore likely that in W7 injected tilapia, the severe effect on androgen production is a result of multiple blockage of the BPG axis, including a local effect in the testis as seen previously *in vitro* (chapter 5).

In vitro incubations of tilapia testis with W7 led to decreased androgen production and activation of cortisol production in the testis (chapter 5). Interestingly, earlier reports have shown that CaM inhibition of bovine adrenocortical cells led to decreased ACTH-stimulated production of cortisol (Niitsu, 1992), suggesting that glucocorticoid production in the adrenals is blocked by W7. In the present study, however, we did not test whether W7 had any effect on adrenal steroid production in tilapia and we have only detected a slight but not statistical significant increase in plasma cortisol levels after W7 administration, which is in contradiction to that obtained *in vitro* in chapter 5. However, these results could have different explanations, namely that (1) cortisol production in the tilapia head kidney is probably not sensitive to this inhibitor, or (2) that the activation of cortisol production in the testis could be strictly local and specific, or (3) that the presence of a functional HPAG axis is counteracting the effect of the inhibitor *in vivo*. Nonetheless, further work is still needed to clarify these results.

In chapter 5, we have suggested that the activity of the 11 β -hydroxylase was probably not regulated by CaM, based on the steroid profiles that we have obtained. Interestingly, at the mRNA level, we did not find a statistical significant difference between control and treated fish in mRNA levels of *CYP11B1*, which suggest that W7 does not

affect either the enzyme activity or the transcriptional levels of CYP11B1. Interestingly, *Arβ* mRNA levels were significantly up regulated by W7 injection. In prostate cancer cells, intracellular calcium levels have been shown to regulate AR expression (Gong et al., 1995). Furthermore, it has also been shown that AR protein isolated from the tumour cell line LNCaP is bound by CaM, and W7 decreases the interaction (Cifuentes et al., 2004). However, in this cell line, the AR that is expressed contains a mutation in the ligand binding domain that affects ligand binding to several ligands (Wang et al., 2002). With the human wild type AR, casodex, an anti-androgen, abolished the CaM-AR interaction while W7 slightly decreased this interaction, suggesting that in wild type AR, CaM is probably not binding AR very efficiently in the presence of a high affinity ligand (Cifuentes et al., 2004). Although, there is no documented link between CaM inhibition and AR transcriptional levels, our study suggest that CaM inhibition does not impair AR transcription levels.

The decreased 11-KT levels after W7 administration in chapter 5 and in the present chapter does not rule out the possibility that there may be an increase in testosterone aromatization within the testis and thus, a shift of androgen to estrogen production. However, in chapter 5 we were not able to detect estradiol in testis incubations. Similarly, in the present chapter we were also not able to detect estradiol in the plasma, suggesting that both *in vitro* and *in vivo*, the decreased androgen production is not due to increased estrogen production. Likewise, in incubations of Atlantic croaker ovaries with two different CaM inhibitors, W7 and trifluoperazine, basal estradiol levels were also not affected suggesting that basal estrogen production was not sensitive to CaM inhibition (Biswas et al., 1998; Benninghoff and Thomas, 2005). Notwithstanding, a lack of effect on enzyme activity does not rule out an effect at the level of gene transcription. Thus, we have decided to study the mRNA expression of the gene encoding the enzyme responsible for estrogen production, CYP19, and also of two estrogen receptors (α and β 2). Interestingly, we found no differences in *CYP19* mRNA levels between control and W7 treated testis suggesting that this inhibitor does not affect the gene transcription. Likewise, the *ERβ*2 mRNA levels were also unaffected by calmodulin inhibition. Interestingly, the *ERα* mRNA levels were significantly decreased in fish treated with W7. These results, suggest that the transcription of these receptors is sensitive to different regulatory factors. In fact, recently, it has been reported that CaM interacts physically with the ER-ERE complex and is involved in the estrogen-induced transactivation of responsive genes in human breast cancer lines (Biswas et al., 1998). Moreover, it has been shown that administration of W7 inhibits the ERα mediated transactivation of an ERE containing promoter but did not affect that of the ERβ (Garcia-Pedrero et al., 2002). Additionally, it has been shown that elimination of calmodulin

binding prevented estradiol from stimulating ER α transcriptional activation in COS-7 transfected cells (Li et al., 2005). Thus, inhibition of ER α transcription in tilapia testis and not of ER β 2 by calmodulin inhibition also suggests that CaM may regulate ER α transcription in the testis and is probably also involved in other molecular actions involving this receptor.

In addition to genes encoding enzymes and receptors involved in sex steroid production and binding, we have also studied the expression of different transcription factors that are known to be involved in the regulation of steroidogenesis and also in male gonadal differentiation and maturation (*DAX1*, *DAX2*, *SF1*, *SOX9.1* and *SOX9.2*).

SF1 has been implicated in determinant steps within male reproductive development. *SF1* null knock-out (K.O.) in male mice results in absence of adrenals and gonads (Luo et al., 1994; Sadovsky et al., 1995). In addition, SF1 is able to regulate the expression of many of the genes that encode steroidogenic enzymes and other regulators of endocrine function (Morohashi and Omura, 1996; Parker et al., 1999). Similarly, *DAX1* is important for male gonad development since mice with mutated *DAX1* gene exhibits male hypogonadism, sterility due to abnormal maintenance of spermatogenesis and also presence of multiple ovarian oocytes in testes (Yu et al., 1998b). *DAX1* and SF1 have been shown to interact physically *in vitro* (Ito et al., 1997; Crawford et al., 1998) and, in addition, they have been shown to share both tissue distribution and regulation of enzymes within steroid hormone production pathway (Zazopoulos et al., 1997; Lalli et al., 1998; Zhang et al., 2001). These studies suggest that the activities of SF1 and *DAX1* converge in steroidogenic tissue development, and that each is required to induce the steroidogenic phenotype. In tilapia testis, W7 administration decreased the *SF1* mRNA levels, although not significantly. In contrast, CaM inhibition led to a significant increase in *DAX1* mRNA levels. These results are interesting since it has been reported Ca²⁺-dependent hCG stimulation of steroidogenesis in mice Leydig tumour cell line (mLTC-1) mediated by increased *SF1* mRNA levels (Manna et al., 1999). However, when mLTC-1 cells were co-transfected with *DAX1*, both basal and Ca²⁺-dependent hCG stimulated transcription of the *SF1* and *StAR* genes were significantly decreased (Manna et al., 1999). Thus, the ratio of *DAX1/SF1* mRNA was significantly decreased by increased Ca²⁺-dependent stimulation of steroidogenesis in mice. In addition, *DAX1* transcription has been shown to be severely down regulated by FSH induced testosterone production and by increased levels of dbcAMP (Tamai et al., 1996), opposite to the upregulation of *SF1* transcription. Altogether these studies in mice suggest that stimulated steroidogenesis in the testis is accompanied by decreased ratio of *DAX1/SF1* mRNA levels, further emphasizing that SF1 and *DAX1* have opposing regulatory functions on steroidogenesis. Interestingly, the ratio of *DAX1/SF1* mRNA levels were significantly increased in treated

fish, which suggests that the shift in ratio of these receptors could also be associated to decreased steroidogenesis and thus to repressive actions of DAX1 in steroid production as described in mice (Tamai et al., 1996). Similarly, the *DAX2* mRNA levels are also significantly increased, suggesting a similar role in gonadal tissue.

We further analyzed the expression of the *SOX9* genes in tilapia testis. The *SOX9* is a transcription factor involved in testis differentiation (Harley et al., 2003). *SOX9* protein contains two nuclear localization signals (NLSs) located at the N and C termini of the HMG box. The NLS at the N termini is in fact a bipartite NLS, to which calmodulin has been shown to bind and regulate nuclear import of SOX proteins, and the C termini NLS is bound by importin β that is also involved in SOX nuclear import (Harley et al., 2003). It has been shown earlier that both CaM and importin nuclear import pathways are required to give maximal *SOX9* nuclear accumulation and may in fact contribute equally (Argentaro et al., 2003). In fact, this is observed in transfection studies using CaM inhibitor W7, since this inhibitor was unable to block nuclear import of *SOX9* by much more than 50%, and the transcriptional activity of *SOX9* was reduced to a lesser extent than was nuclear import (Argentaro et al., 2003). Thus, this study shows that although calmodulin inhibition reduces nuclear import, the *SOX9* protein can still be transported by other molecules into the nucleus and, suggests that its transcriptional activity in the nucleus is probably not affected by calmodulin inhibition. In tilapia testis, calmodulin inhibition did not affect *SOX9.1* and *SOX9.2* mRNA levels in the testis, further suggesting that although CaM may have a role in nuclear import, it is apparently not affecting *SOX9.1* and *SOX9.2* gene transcription, as seen in mammals.

In tilapia, calmodulin inhibition blocked testosterone and 11-KT production, but in chapter 5, we have also shown that *in vitro*, this decrease is accompanied by decreased androstenedione levels. However, we did not rule out that these decreased steroid levels are not due to accumulation of 11 β -hydroxyandrostenedione. This possibility is supported by studies showing that increased 11 β -hydroxyandrostendione levels and not 11-hydroxytestosterone levels, inhibit both ovarian and brain aromatase activity (González, 2003), suggesting that increases in this steroid could have a major impact on gonadal sex differentiation. Thus, we have decided to study the mRNA expression of the gene coding for the enzyme aromatase (*CYP19*) in tilapia testis. Although we have detected aromatase mRNA levels in both control and W7 treated fish, we did not find any significant effect of W7 administration on *CYP19* transcription levels. Thus, although we cannot discard the possibility that 11 β -hydroxyandrostendione may be accumulating in result of CaM inhibition, we did not detect any influence at the transcription levels of *CYP19* in the testis.

Interestingly, the mRNA expression of the genes analysed in tilapia testis after CaM inhibition are remarkably similar to those found in gonadal sex reversing aromatase

KO (ArKO) females. In ArKO females the increase in *DAX1/SF1* ratio in the gonads was accompanied by decreased *ERα* mRNA levels (Britt et al., 2004). Likewise, in tilapia, calmodulin inhibition led to decreased *ERα* levels and to an increase in *DAX1/SF1* ratio. *DAX1* has been shown to interact with estrogen receptors at the protein level, although interaction with *ERα* was ligand dependent and *ERβ* was not (Zhang et al., 2000). Interestingly, *ERα* has also been shown to bind to classical SF1 response elements (Vanacker et al., 1999), as those found in the *DAX1* promoter, suggesting that this receptor can also function as a regulator of *DAX1* transcription. Thus, these results, suggest that the CaM signalling pathway is able to affect not only steroid production at the enzyme levels as it is also able to influence gene transcription and/or transcriptional activity of different targets that ultimately will contribute to the regulation of steroid production in the gonads.

Altogether, the results obtained in this chapter suggest that the Ca^{2+} -CaM signalling pathway is determinant for androgen production in the testis *in vivo*. In addition, we have shown that although CaM inhibition had a significant effect on steroid production, it did not affect the transcriptional levels of either *CYP11B1* or *CYP19*, suggesting that its effect is probably only on the enzyme activity. Moreover, we have shown that CaM inhibition increases the ratio of *DAX1/SF1* mRNA levels, suggesting opposing roles of these transcription factors on fish steroid production as described in mammals. Also, CaM inhibition was shown to have a significant effect on the transcriptional levels of *DAX2*, *ARβ* and *ERα*, but not of *ERβ2*, *SOX9.1*, and *SOX9.2*. In concert, the work presented in this chapter corroborates the hypothesis of a common gene network of CaM signalling and *DAX1* in steroid production in male fish gonads.

Chapter VI

General discussion

In this thesis we have isolated 3 different members of the *Nr0B* family in the European sea bass (*Dicentrarchus labrax*), named *sbDAX1*, *sbDAX2* and *sbSHP* (chapter 2 and 3). Analysis of gene structure and sequence conservation has shown that this family of genes may have arisen from a common ancestral gene that has undergone two rounds of gene duplication in fish species, one common to vertebrates that has yielded an ancestral *DAX* gene and a *SHP* gene, and a subsequent specific round of genome duplication that has yielded duplicate *DAX* genes (*DAX1* and *DAX2*) and duplicate *SHP* (*SHP1* and *SHP2*) in fish. In sea bass, however, we were not able to determine whether the *SHP* has also a duplicate gene in its genome, as we have only identified 1 gene to date. Nonetheless, future work will most likely answer this question.

All sea bass *Nr0B* family members identified in this thesis were shown to be highly similar to homolog genes characterized in other vertebrates. The main features identified in mammal *DAX1* and *SHP* are also present in sea bass homolog genes, namely the presence of putative LXXLL motifs, conserved transcriptional silencing domains and AF-2 cores. Although we did not test whether these elements are functional in fish, the high sequence conservation suggests that they are probably used to mediate similar molecular interactions as described in mammalian (chapter 2 and 3). Thus, future work should be done in order to assess whether these genes also use these motifs for receptor interaction. In addition, the difference in LXXLL motif number and conservation between the duplicate *DAX1* and *DAX2* genes in fish also raises the question of whether these differences are somehow related to different physiological roles acquired by these receptors after gene duplication.

Interestingly, the main objective of this thesis was to determine whether *DAX1* was involved in fish sex determination. In this respect, the choice of using sea bass as a study model has proven to be quite interesting. To date, the only factors that have been shown to be effective in skewing the sea bass sex ratios towards either female or male differentiation is temperature and exogenous steroid administration (Pavlidis et al., 2000; Blazquez et al., 2001; Koumoundouros et al., 2002). Temperature treatments, applied during the first 60-90 dph of development, can produce sex biased populations. In this thesis, we have studied the mRNA expression of the three *Nr0B* members in larvae grown at either a feminizing or at a masculinising temperature, which have yielded female dominant and male dominant populations, respectively. It has been a few years since the effect of temperature on sea bass sex ratios was determined, and to date, there are still no explanations for this effect of temperature on sex ratios. However, one of the hypotheses that have been suggested by different researchers is that temperature may be influencing the expression of either enzymes/steroid receptors or of sex determining

genes during the early development. In sea bass, steroid enzymes CYP19 and CYP11 have been shown to be expressed during the TSD, but only at residual levels, suggesting that the effect of temperature was likely on upstream genes involved in earlier events of gonad formation (Socorro et al., 2007). As such, the *DAX1* and *SOX9* genes have been considered possible candidate targets for temperature modulation in TSD. Interestingly, we have found that there is a highly dimorphic pattern of expression of *sbDAX1*, *sbDAX2*, *SOX9.1* and *SOX9.2* mRNAs, between larvae grown at either 15 or 21°C. Indeed, between 32 and 60 dph, low temperatures appear to suppress gene expression, when compared to that seen at high temperature (chapter 3). These results are very interesting since both *DAX1* and *SOX9* are determinant of sex in other vertebrate species. Thus, the results presented in chapter 2 and 3 suggest that temperature exerts its effect on sea bass sex determination through directly or indirectly altering gene expression at early stages of development and also that *DAX1*, *DAX2*, *SOX9.1* and *SOX9.2* most likely are part of the upstream sex determining gene cascade in sea bass. Nonetheless, regardless of the effect of temperature on gene transcription, it is still necessary to determine whether temperature is influencing gene transcription directly or as a consequence of its influence on other physiological processes namely in growth rate. Thus, future work with gene promoters will likely answer if temperature is affecting transcription directly. Interestingly, we have found that the transcriptional levels of *sbSHP* are also strongly influenced by temperature (chapter 3), suggesting that this gene may be a novel candidate sex determining gene in fish.

Further support for a role of *DAX1* in sex determination has been obtained from chapter 4, through the analysis of *DAX1* promoter conservation. Indeed, we have identified the sea bass 1kb upstream promoter sequence, and found several conserved putative binding sites for regulatory elements known to regulate the mammal *DAX1* transcription. Moreover, despite the high conservation in these binding sites, we were surprised by the large conservation in promoter architecture, as demonstrated by the presence of common frameworks composed of 3-5 TF binding sites that are present in most of the fish and mammal *DAX1* promoters, within the same order and distance. Indeed, these results, together with the observed sequence and motif conservation in protein structure between fish and mammalian (chapter 2), suggest that the transcriptional regulation of fish *DAX1* is probably very similar to that described in mammalian, although at the level of the protein structure they may have differed more rapidly between species. In addition, when we searched for annotated gene promoters with common promoter architecture, we have found that several genes involved in vertebrate sex determining pathway, steroid production and regulation, liver function and metabolism, and in cartilage and bone development and homeostasis, also possess common frameworks, suggesting

that they are within the same regulatory networks as vertebrate *DAX1* genes. Interestingly, the mammalian *DAX1* gene has been shown to be involved or expressed in similar tissues and/or within these physiological processes, further suggesting that *DAX1* genes most likely have conserved functions. Altogether, the results obtained in chapter 4 have suggested different gene networks within which the fish *DAX1* may be involved, and therefore, have highlighted a possible role in liver function and development and in bone development. Thus, this thesis has highlighted the need of investigating the role of *DAX1* in different research areas aside the field of reproduction and steroid production in fish. Indeed, the development of different KO and gene overexpression models in sea bass (*DAX1*KO, *DAX2* KO and *DAX1/DAX2* KO) should help us to determine whether *in vivo*, these nuclear receptors are involved within the different processes identified in this thesis.

One of the gene networks that we have identified in chapter 4 and that we have decided to focus on in the last two chapters (5 and 6) refers to the role of *DAX1* in steroid production in gonad cells. Indeed, different studies had shown that mammalian *DAX1* and *SF1* are key regulators of steroid production in the gonads. However, although most studies have demonstrated the effect of gonadotrophin stimulation of gonadal steroid production, it is still not known what the role of *DAX1* is within this regulatory process. Indeed, gonadotrophin regulation of steroid production has been shown to be achieved through activation of the CaM and cAMP-PKA signalling pathways, either through their effects on steroid enzyme function or on *SF1* activity and transcription in endocrine tissues. However, there is still no information on the effect of the modulation of these signalling pathways on *DAX1* activity or transcription. Interestingly, promoter analysis with vertebrate *DAX1* promoters have suggested that *DAX1* transcription is co-regulated with CaM and other calcium binding proteins, suggesting that *DAX1* transcription is may be affected when Ca^{2+} -CaM signalling pathway is modulated. In tilapia, both *in vitro* incubations and *in vivo* administration of a CaM inhibitor, W7, has resulted in suppressed androgen production. In addition, we have also shown that *in vitro*, modulation of androgen production by cAMP-PKA activation is dependent on the activation of CaM signalling pathway since, forskolin stimulated androgen production was effectively blocked by calmodulin inhibition. Interestingly, in mammalian gonads, gonadotrophin stimulation of cAMP-PKA signalling pathway has been shown to result in phosphorylation of *SF1*, enhanced *SF1* activity and increased steroid enzyme activity, and also to decreased expression of *DAX1* suggesting that modulation of cAMP and/or Ca^{2+} levels influences *DAX1* transcription. Interestingly, we have also found that blocking the CaM *in vivo* resulted in suppression of steroid production and in increased ratio of *DAX1/SF1* mRNA and *DAX2* mRNA levels, suggesting that suppression of CaM signalling down regulated *SF1* mRNA levels. Thus, these results suggest that, *DAX1* and *DAX2* mRNA levels are

probably repressed by increased CaM levels, further suggesting that calmodulin and *DAX1* are involved within the same network as suggested in chapter 4, by the promoter analysis. Nonetheless, these results also raise different questions, namely whether the effect of CaM inhibition on *DAX1* and *DAX2* transcription is due to a direct effect on gene transcription or if, as in cAMP modulation, the lack of activation of SF1 protein is abolishing its repressive effect on *DAX1* transcription and thus allowing it to increase the transcription levels. Thus, future studies with *DAX1* promoter regulation should allow answering these questions.

In conclusion, although we did not obtain conclusive data supporting a role for fish *DAX1* and *DAX2* genes in either male specific or female specific sex determination, we did find a link between temperature and *DAX1* and *DAX2* transcription levels during the TSD supporting the view of a role of these genes within the early gene cascade involved in TSD. In addition, we have also shown that Ca^{2+} -CaM signalling pathway is not only involved in the modulation of steroid production within male fish gonads as it is also involved directly or indirectly in the modulation of both *DAX1* and *DAX2* gene transcription within the gonads.

CHAPTER 8

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