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DOES THE INTERRENAL INFLUENCE SEX DIFFERENTIATION IN SEA BASS, *Dicentrarchus labrax*?

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Ruta Sofia Tavares Martins

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ABSTRACT

Sea bass *Dicentrarchus labrax* is one of the most important cultured species in Mediterranean aquaculture. This species remains sexually immature most of the first year of life, and at the time of marketing (2 years old), females are 18-40% heavier than males. However, in cultured populations, it is frequently reported skewed sex ratios in favour of males (reaching 70-99%), and thus, the acquisition of all-female stocks is an attractive option for sea bass aquaculture. The underlying hypothesis of this work is that in intensive culture, the sea bass interrenal tissue produces corticosteroids in response to stress, and together with them an excess of adrenal androgens shifting the normal androgen/ estrogen ratio and thus leading to gonadal masculinization. Thus, blocking cortisol production with an antagonist (Dexamethasone) during the androgen sensitive period would most likely decrease the androgen levels and thereby the sex ratios would be altered.

Administration of 75 mg Kg^{-1} food dexamethasone to fish during the period of sexual differentiation did not alter the sex ratios in sea bass (60-65% males; 35-40% females), however, 150 mg Kg^{-1} food dexamethasone induced some alterations to the normal development of the gonads. In this respect, 7% of the fish had testicular tissue with scattered intra testicular oocytes and 4% of the fish were still undifferentiated at 10 months of age. However, the doses of dexamethasone administered to the fish had significant side- effects on the normal growth of the fish. Furthermore, all stress experiments performed failed to show a correlation between the elevated levels of cortisol and androgen levels (testosterone and 11-ketotestosterone) in sea bass. Overall, no conclusive data was obtained to establish a direct role of the interrenal tissue corticosteroidogenesis on sexual differentiation.

Two steroidogenic enzymes (*CYP* 19 and *CYP* 11B1) and four steroid receptors (ERα, ERβ1, ERβ2 and ARβ) were isolated in sea bass. In sexually differentiated fish, the expression of *CYP* 19, ERβ1 was higher in the ovarian tissue than in the testicular tissue. On the contrary, the expression of *CYP* 11B1, ERβ2 and ARβ was higher in the testicular tissue than in the ovarian tissue. The expression of ERα was never detected in either gonad.

During the androgen sensitive period, the expression of *CYP*11B1 and ARβ was high but decreased towards the end of this period. *CYP* 19 and ERβ2 were initially

weakly expressed but at the end of this period, the expression of these genes increased. ERβ1 presented a high dispersion of expression throughout this period. The expression of ERα was never detected. Moreover, the expression of *CYP*11B1 and ARβ was inversely correlated to the expression of ERβ2. From all the genes isolated, only the expression of ARβ presented a dimorphic pattern of expression during the androgen sensitive period.

Altogether: (1) no correlation between cortisol and androgens could be found in response to stress; (2) administration of dexamethasone induced some alterations on the normal differentiation of the gonads, although no conclusive effect could be seen on the sex ratios; (3) during the androgen sensitive period there was a negative correlation between the expression of *CYP*11B1 and ARβ with the expression of ERβ2. From all the genes studied only the ARβ presented a dimorphic pattern of expression, which points out that this gene most likely possesses an essential role in sexual differentiation in sea bass.

I- INTRODUCTION

Sea bass *Dicentrarchus labrax* is one of the most important cultured species in Mediterranean aquaculture. With the enormous expansion of fish culture in the past years, it has become evident that there is a necessity to enhance the expression of the associated morphological and physiological characteristics that would be advantageous under certain culture strategies. In this respect, it is desirable to eliminate the sex that shows less growth and to prevent precocious sexual maturation that is associated to weight loss and to decreased flesh quality (Piferrer *et al.*, 1989).

Sea bass remains sexually immature most of the first year of life. Most males reach puberty in the second year of life, while females reach puberty in the third year (Blázquez *et al.*, 1995). Thus, males divert energy resources into gonad development, while females use those resources for somatic growth. As a result, females are 18-40% heavier than males at the time of marketing (at 2 years of age) (Blázquez *et al.*, 1995; Pavlidis *et al.*, 2000). Since females do not mature before the time of marketing, and grow faster than males, the acquisition of all-female stocks is an attractive option for sea bass aquaculture.

In natural populations, predominance of female or male fish has been equally reported in sea bass (Chatain *et al.*, 1999). In cultured populations, however, skewed sex ratios in favour of males are frequently reported (reaching 70-99%) (Blázquez *et al.*, 1998). For this reason several studies have been conducted aiming to understand the factors affecting sexual differentiation in this species, and to achieve more balanced sex ratios or even female-dominant stocks.

Sea bass lack heteromorphic sex chromosomes and ploidy manipulations failed in modifying the sex ratio (Pavlidis *et al.*, 2000; Chatain *et al.*, 2000). Similarly, attempts to obtain a molecular probe to identify the genetic sex in this species have also failed (Ollevier *et al.*, 1998).

In this species, size rather than age, has been shown to be a critical marker of the timing of sex differentiation (Blázquez *et al.*, 1998). Nonetheless, the failure to identify sex during the early developmental stages does not allow to determine whether within a certain population, individuals become males because of their smaller growth, or smaller because of their maleness (Pavlidis *et al.*, 2000).

The high diversity of reproductive strategies in fish, together with this diversity of sex-influencing factors, explains why no pattern of sexual differentiation can be

generalized for fish. Sex steroids, however, are involved both in triggering and/or throughout the differentiation of the gonads in all fish species (Yamamoto, 1968).

It has been shown that in sea bass, the critical period during which the gonads are still undifferentiated but exhibit increased sensitivity to exogenous steroids treatments is located between days 57 and 137 post-fertilization (Pavlidis *et al*., 2000). The critical period for androgen-inducible masculinization is between 96 and 126 days post-fertilization (Blázquez *et al*., 2001). Thus, administration of androgens during this period of development results in all-male populations (Chatain *et al*., 1999; Blázquez *et al*., 2001; Blázquez *et al*., 1995). Moreover, oral administration of estrogens (estradiol) has been shown to result in all-female populations in sea bass (Chatain *et al*., 2000).

Several attempts have been made to show that environmental parameters used under rearing conditions are responsible for the excess of males in sea bass aquacultures. So far, it has been shown that the period of ontogenesis in sea bass seems to coincide also with a thermosensitive period and that the sex ratios can be manipulated by controlling water temperature (Pavlidis *et al*., 2000; Bláquez *et al.,* 1998). However, results are contradictory and suggest different effects of the same range of temperatures on sex ratios, depending on the stage of development of the fish. In this respect, eggs incubated at low temperatures (15°C) will result in female-biased populations (Pavlidis *et al*., 2000), while fish larvae (57 days post-hatching) incubated at the same temperature will result in male-biased populations (Bláquez *et al.,* 1998). Even though it is still not possible to establish how temperature influences sex ratios in this species, a recent report has given evidence for a genotype temperature interaction (Saillant *et al*., 2002). Thus, breeding fish (both female and male fish), raised at high temperatures strongly affect the sex ratios of the progenies, by increasing the proportion of females (Saillant *et al*., 2002).

Despite the possible effects of temperature on sex differentiation, it is possible that other environmental factors in the fish farm may be influencing the sex ratios. These may include the stocking density, amount of food, confinement and social interactions. Most of these environmental parameters elicit stress responses, which are patent in the elevated cortisol levels in several captive fish species, including in sea bass. In addition, acute forms of handling stress, such as those associated with the routine hatchery procedures of grading, transportation and artificial stripping, also contribute to the elevation of plasma catecholamines and corticosteroids in captive fish (Pickering, 1981; Barton and Iwama, 1991).

Aim of the study

The underlying hypothesis of this thesis is that in intensive culture, the sea bass interrenal tissue produces corticosteroids in response to stress, and together with them an excess of adrenal androgens shifting the normal androgen/ estrogen ratio and thus leading to gonadal masculinization. In order to test this hypothesis: (1) dexamethasone, a synthetic glucocorticoid was used to block the Hypothalamus-Pituitary-Interrenal (HPI) axis production of cortisol and possibly androgens, starting at the period of highest sensitivity to androgens, until gonads were differentiated; (2) sea bass were exposed to either confinement or handling stress experiments in order to test if the elevated cortisol levels are accompanied by the elevation of androgen levels; (3) In addition, a study was conducted of expression of mRNA for steroidogenic enzymes involved in androgen and estrogen production as well as of steroid receptors covering the period of sex differentiation.

1.1- Sex determination

Fish, amphibians and reptiles, exhibit several mechanisms of sex determination. No simple gonadal sex determination (GSD) model can be generalized for fish and, even though some species display simple heterogametic models (XX/XY or ZW/ZZ), other variations can be found, including male and female heterogamety within the same species, and hermaphroditic species (Scherer, 1999).

The molecular mechanisms involved in GSD have been intensively studied in mammals and increasing information is now becoming available for non-mammalian (birds, reptiles, amphibians, fish) and invertebrates species (e.g. insects, nematodes).

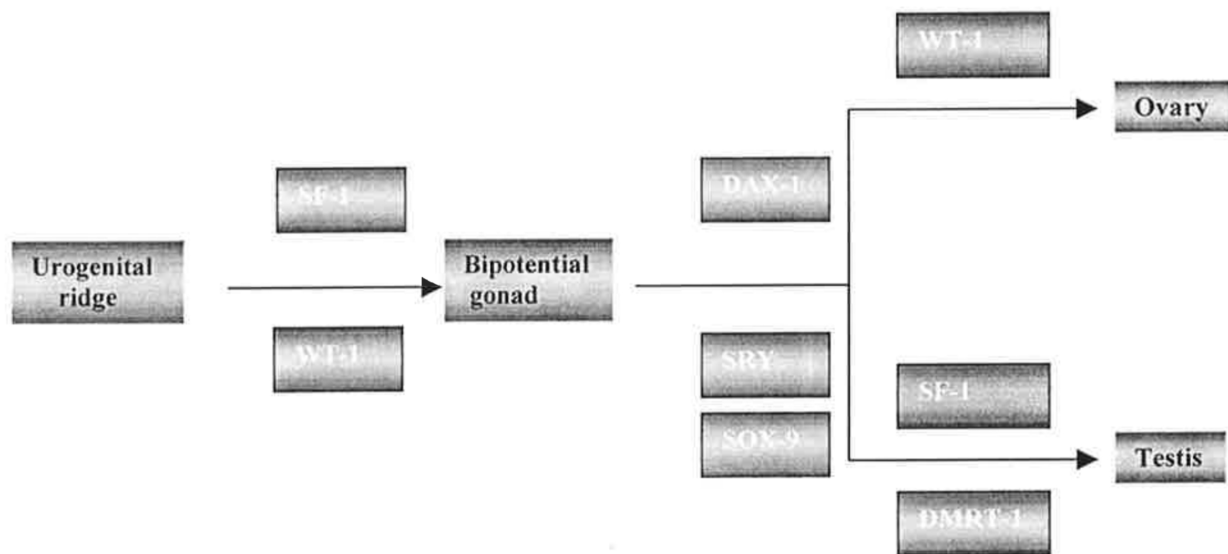


Figure 1- Molecular events during gonad development and sex induction in mammalians. Effector genes or gene products are shown in black boxes.

A number of genes involved in mammalian gonad development have been identified. As shown in figure 1, these can be roughly sub-divided in three classes based on their order of action. Firstly, the genes required for the formation of the sexually undifferentiated gonad, such as steroidogenic factor-1 (SF1) and Wilms tumor-1 (WT1). Secondly, genes such as the testis- determining factor on the Y chromosome; SRY, SOX- 9 and possibly Dax-1, which prepare the gonad to differentiate either into male or female. And thirdly, genes that promote male or female differentiation of the gonad once its sex has been determined, including SF1, DMRT-1 and WT1 (Raymond *et al.*, 1999; Parker *et al.*, 1999).

Although vertebrates exhibit an array of sex determining mechanisms, and even though the GSD upstream pathways can diverge among different taxa, it is becoming apparent that some downstream sex determining genes are functionally similar in several species. For instance, DMRT-1, a gene involved in testicular differentiation, is highly conserved both at structural and functional levels, from invertebrates to higher vertebrates (Raymond *et al.,* 1999; Smith *et al.*, 1999; Raymond *et al.*, 1998; Raymond *et al.*, 2000; Marchand *et al.*, 2000). This supports the hypothesis that there may be some degree of functional evolutionary conservation of sex determining genes across taxa (Ottolenghi and McElreavey, 2000).

In fish, little is known on the molecular mechanisms regulating sex determination. Nonetheless, it has been hypothesized that sex differentiation is controlled ultimately by specific sex-determining genes (Baroiller *et al.*, 1999). In contrast with other taxa, there is an enormous plasticity in gonadal development, and interactions between the genome and internal and/or external factors can influence gonadal differentiation or may even lead to complete sex reversal (Pavlidis *et al.*, 2000).

1.2- Sex differentiation

Like all vertebrates, fish go through a sex-neutral stage during early embryonic development. Prior to sexual differentiation, the ovaries and testes cannot be distinguished and therefore are called bipotential or undifferentiated gonads. These bipotential gonads arise from the urogenital ridge, a region that interestingly also contributes cell lineages to the adrenal cortex (the functional equivalent of interrenal or head kidney in fish), kidney and gonads (Parker *et al.*, 1999; Scherer, 1999).

In teleosts, only a single primordial cortex appears to be involved in the ontogeny of both testes and ovaries (Hoar, 1961), but the timing of gonadal development differs among species as shown in Table 1.

Table 1- Timing of gonadal development in different fish species. (dph- days post hatching).

Group/Species	Age at gonadal differentiation	References
Perciformes		
Tilapia zillii	15 dph	Yoshikawa & Oguri, 1976
Oreochromis niloticus	23-26 dph	Hines *et al.*, 1999
Dicentrarchus labrax	9 months	Blázquez *et al.*, 1998
Salmoniformes		
Onconrhynchus mykiss	2-3 months	Liu *et al.*, 2000
Cypriniformes		
Cyprinus carpio	2-4 months	Davies & Takashima, 1980
Mugiliformes		
Mugil cephalus	7-14 months	Chang *et al.*, 1995
Siluriformes		
Ictalurus punctatus	3-4 months	Patiño *et al.*, 1996

In some species, however, it is not accurate or even possible to consider a time scale for gonadal differentiation since it has been shown that sexual differentiation is more closely related to fish length than to fish age, as shown in Table 2, emphasizing that it is a developmental process.

Table 2- Relative size necessary for gonad differentiation.

Group/Species	Length at gonadal differentiation	References
Atheriniformes		
Odontesthes bonariensis	11-18 mm	Strüssmann *et al.*, 1997
Myxiniformes		
Eptatretus stouti	20-33 cm	Gorbman, 1990
Perciformes		
Cichlasoma citrinellum	52 mm	Francis and Barlow, 1993

A broad range of literature is available on the control of gonadal sex differentiation in gonochoristic and hermaphroditic (protandric, protogynic and synchronous hermaphroditic) fish species and it is well established that in fish, as in other vertebrates (e.g. reptiles), there is an environmental as well as endocrine control of the phenotypic sex of the gonads (Baroiller *et al.*, 1999). Consequently, the genotypic and phenotypic sex in fish may not necessarily coincide.

1.2.1- Environmental sex differentiation

Several environmental factors have been tested for their influence on sex differentiation in fish as shown in figure 2. From those we can highlight stocking density and temperature by the fact that fish reared in aquaculture systems are often subjected to extremely high stocking densities and extreme temperatures (both high and low), which usually do not occur in natural conditions.

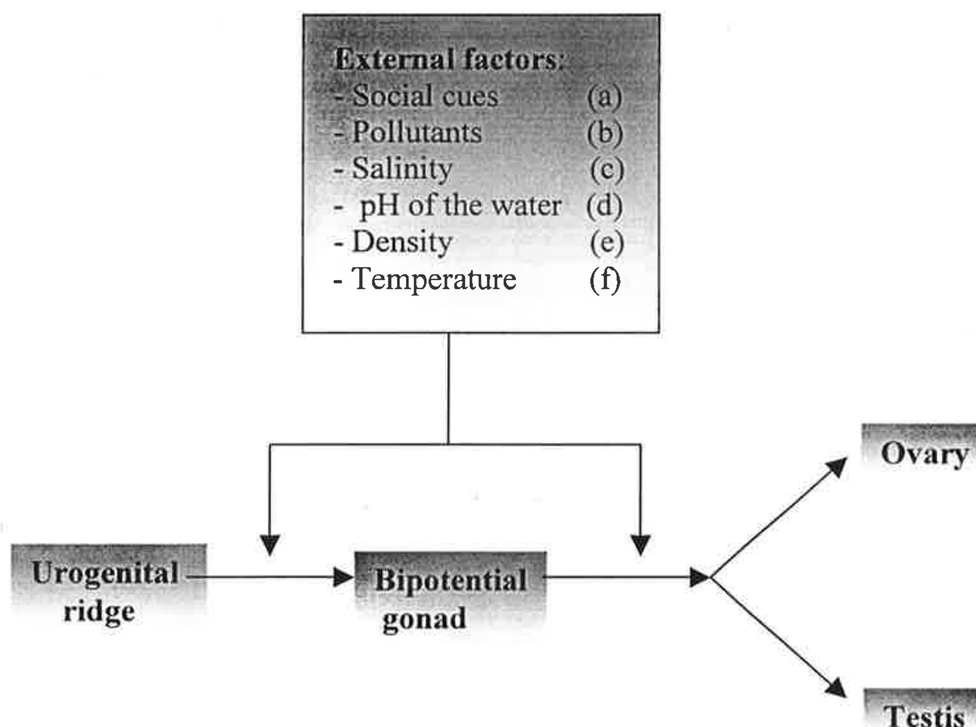


Figure 2- Environmental factors suggested to influence gonadal differentiation. (a) Badura and Friedman, 1988; Francis and Barlow, 1993; (b) Craig *et al.*, 1996; (c) Abucay *et al.*, 1999; (d) Römer and Beisenherz, 1996; (e) Baroiller *et al.*, 1999; (f) Blázquez *et al.*, 1998.

Among the environmental factors involved in sex differentiation, temperature is the most studied. Thus, rearing temperature has been shown to influence sex differentiation in several vertebrates such as amphibians (Baroiller *et al.*, 1999), reptiles (Pieau *et al.*, 1999; Pieau *et al.*, 1998; Dorizzi *et al.*, 1996) and fish (Pavlidis *et al.*, 2000; Blázquez *et al.*, 1998; Craig *et al.*, 1996; Abucay *et al.*, 1999; Strüssmann *et al.*, 1997; Patiño *et al.*, 1996).

In most fish species with thermo labile sex-determination (TSD), interactions between environmental factors and genotype have been strongly suggested. Thermo sensitivity in fish resembles the case of amphibians in which, genetic sex determination governs sex differentiation at ambient temperatures, whereas extreme temperatures (both low and high) strongly affect gonadal sex differentiation (Baroiller *et al.*, 1999; Strüssmann *et al.*, 1997; Patiño *et al.*, 1996; Craig *et al.*, 1996).

Several studies have suggested a link between sex steroids and the mechanism of TSD. It has also been suggested that there may be a relationship between the critical period for response to exogenous sex steroids and the critical period for response to temperature in fish (Blázquez *et al.*, 1998). Also, administration of steroid hormones, steroid antisera, and steroid antagonists has proven to be successful in disrupting normal gonadal differentiation in TSD species (Smith and Joss, 1994; Merchant- Larios *et al.*, 1997).

Likewise, most studies performed in reptiles point out that temperature exerts its action on the metabolic route(s) that lead to the synthesis of sex steroids. Thus, it has been suggested that the ratio of estrogens to androgens, or the production of estrogens alone, may be temperature sensitive and may mediate the influence of temperature on gonadal sex differentiation in TSD species (Smith and Joss, 1994).

Experiments carried out with different models such as reptiles (Pieau *et al.*, 1999; Desvages and Pieau, 1992; Chardard *et al.*, 1995; Rhen and Lang, 1994; Smith and Joss, 1994), birds (Villalpando *et al.*, 2000) and fish (Chang *et al.*, 1999, Cotta *et al.*, 1999; Kwon *et al.*, 1999) have demonstrated the implication of estrogens and the key role played by the enzyme complex that converts androgens to estrogens- cytochrome P450 aromatase- in ovary differentiation during TSD. This hypothesis has gained further support since it has been shown that in most TSD species, the sensitive period in which changes in gonadal aromatase activity can be induced by temperature shifts corresponds to the thermo sensitive period for gonadal differentiation (Chardard *et al.*, 1995).

In fish, the effects of other environmental factors such as pH, salinity, density and social interactions have also been shown to affect gonadal development in different species. An influence of water pH on sex differentiation, either alone or through interactions with temperature, has been reported in a number of species, e.g. *Xiphophorous helleri* (Rubin, 1985 Copeia), *Poecilia melanogaster* (Römer and

Beisenherz, 1996) and some species of *Apistogramma* (cichlids) (Römer and Beisenherz, 1996).

Density and/or social interactions within the fish population have also been shown to affect the sex ratio in several species, e.g. *Cichlasoma citrinellum* (Francis and Barlow, 1993); *Anthias squamipinnis* (Fishelson, 1970); *Labroides dimidiatus* (Shapiro, 1980); *Gonostoma bathyphylum* (Badcock, 1986) and *Betta splendens* (Badura and Friedman, 1988).

1.2.2- Endocrine sex differentiation

The levels of endogenous sex steroids change during embryonic and early development of teleosts (Hines *et al.*, 1999). Generally, developing embryos exhibit relatively high levels of sex steroids just after fertilization, which decline during fry emergence (Khan *et al.*, 1997; Chang *et al.*, 1995). This suggests that the temporal change in steroid levels during development reflect initial metabolic processing of maternal steroids by embryos. Furthermore, it has been shown that in teleost species, embryos have the capability of conjugating steroids, thus providing a means of elimination of some compounds (Iwata *et al.*, 1994; Stouthart *et al.*, 1998).

Early exposure of embryos or larvae to estrogens or androgens has proven effective in manipulating the phenotypic gender of the gonads (Patiño *et al.*, 1996; Kime, 1978; Gilling *et al.*, 1996; Blázquez *et al.*, 1999; Chatain *et al.*, 1999). Such studies indicate that steroid hormone production is not only essential for the normal expression of gender, but also that the early embryos of fish appear to have the ability to synthesize and metabolise steroids. In fact, the presence of steroid synthesizing enzymes has been demonstrated during early life stages of several fish species, namely in guppy (*Poecilia reticulata*) and in rainbow trout (*Oncorhynchus mykiss*) (Yeoh *et al.*, 1996; Feist and Schreck, 1996).

It has been hypothesized that gonadal sex is determined by the local androgen to estrogen ratios with relatively higher or lower ratios yielding testicular or ovarian development, respectively (Hines *et al.*, 1999). Considering this hypothesis, a more or less clear sexual dimorphism of plasma profiles of sex steroids around the period of sexual differentiation would be expected. There are only a few reports of the profiles of sex steroids during the period of sexual differentiation in fish, but it is evident that there is no clear pattern, which can be generalized. It has been shown that in tilapia (*O.*

niloticus) (Nakamura and Nagahama, 1989) and coho salmon (*Onconrhyncus Kisutch*) (Feist *et al.*, 1990), the levels of either testosterone or testosterone plus 11-ketotestosterone, respectively, reflect the process of sexual differentiation. In contrast, in the grey mullet (*M. cephalus*), no clear sexual dimorphism of plasma profiles in testosterone or estradiol was found during the period of sexual differentiation (Chang *et al.,* 1995).

Assuming that steroid hormones are critical for directing initial sex differentiation, steroid-producing cells should be apparent prior to morphological differentiation of the gonad. One possibility is that the adrenal gland can function as the principal site of steroid biosynthesis during sexual differentiation, long before gonad differentiation. This is clear in several reptile species such as the tropical lizard *Calotes versicolor*, the sea turtle *Lepidochelys olivacea*, *T. scripta* and *Crocodylus porosus* (Doddamani, 2000). In fish, however, there is no individualized adrenal gland, but homologous functions are carried out by a specialized tissue- the interrenal or head kidney. Several reports have shown that fish interrenal tissue is able to synthesize several steroids, which are typically secreted in the gonadal tissue. In fact, in some fish (e.g. African catfish *Clarias gariepinus*), most steroids usually synthesized in the testes are still detected in blood plasma after castration, as they can also be synthesized in the interrenal and other tissues (Vermeulen *et al.,* 1995).

Furthermore, in several fish larvae such as tilapia *Oreochromis niloticus* (Hines *et al.*, 1999), and rainbow trout *Onchorhynchus mykiss* (Susuki *et al.*, 1997), the hypothalamus-pituitary-interrenal axis (HPI) is already functional at early stages, consistent with the hypothesis that a steroid biosynthetic capacity precedes gonadal differentiation.

Several reports have established a relationship between hormones of the HPI axis (which are released during stress) and the hypothalamic- pituitary- gonadal (HPG) axis (Rivier and Rivest, 1991; Pickering *et al.*, 1987; Huang *et al.*, 1999; Teitsma *et al.*, 1998; Carragher and Sumpter, 1990; Pankhurst *et al.*, 2000). At the level of the gonads, adrenal corticoids, pro-opiomelanocortin (POMC)-like peptides, and corticotropin-releasing factor (CRF) are reported to interfere with the stimulatory action of gonadotropins on sex steroid-producing cells and thus alter the reproductive function. However, despite the well-established relationship between the HPI and HPG axis in fish, little is known on the influence of adrenal steroids on sex differentiation in vertebrates.

In humans, several cases of sexual ambiguity have been reported due to malfunction of the adrenals. An example is the Congenital Adrenal Hyperplasia (CAH) syndrome. CAH is a form of adrenal insufficiency in which the enzymes involved in the production of adrenal corticosteroid hormones, is deficient: because cortisol production is blocked, the adrenal gland overproduces androgens, which lead to the virilization of female foetuses (Forest and David, 1992). This syndrome results from deficiencies of the enzymes 21- hydroxylase (Forest and David, 1992; Schawab *et al.*, 2001), 11- beta hydroxylase (Bouchard *et al.*, 1989) or 3β-HSD (Pang, 2001).

In rats, cortisol administration during pregnancy induced a shift of the differentiation of the foetus female genital tract into the male direction (Roland *et al.* (1977). In fish, however, few studies have shown effects of adrenal steroids in the process of sex differentiation. High dosage treatments of rainbow trout with cyanoketone (a 3β-HSD inhibitor) resulted in a significant shift of the sex ratios towards the male differentiation. This inhibitor also increased the 3β -HSD activity in the interrenal tissue, but not in the gonads, suggesting that stimulated steroidogenesis in the interrenal tissue, could lead to dramatical changes in the sex ratios (van den Hurk and van Oordt, 1985).

In vitro experiments, namely in rainbow trout *Onconrhyncus mykiss*, *Esox lucius* and perch *Perca fluviatilis* have shown that adrenal corticosteroids can be metabolised by 11β-HSD into 11β- hydroxyandrostenedione and 11β- ketoandrostenedione, which are known to be potent masculinizing steroids (Goswami *et al.*, 1985; Kime, 1978). Furthermore, administration of cortisol and cortisone to rainbow trout fry has been shown to skew the sex ratio in favour of males, which suggested that the two corticosteroids could have been converted into 11-oxygenated androgens (Schulz, 1986). It has been hypothesized that 11-oxygenated androstenedione derivatives which are present in large amounts within the free steroid metabolites produced by newly hatched larvae in several fish species, e.g. Artic Charr (Khan *et al.*, 1997), catfish *Clarias gariepinus* (Khan *et al.*, 1997) and Asian sea bass (*Lates calcarifer*) (Guigen *et al.*, 1995), are involved in sustaining the differentiation and early development of the testis in fish (Baroiller *et al.,* 1999). This could be a possible pathway through which adrenal corticosteroids may influence sex differentiation.

1.3- Interrenal tissue

Several experimental approaches over the last decades have established that the pituitary hormone adrenocorticotrophin (ACTH) is the principal regulator of the interrenal tissue (Bradford *et al.*, 1992). ACTH is not only responsible for maintaining interrenal homeostasis in adult animals (acting as a growth factor and preserving the differentiated state of the interrenal) and promoting interrenal development in larvae but it is also involved in increasing the levels of precursors for steroid synthesis and the synthesis and activation of enzymes leading to the formation of steroid hormone molecules namely mineralocorticoids, glucocorticoids and androgens (Bülow *et al.*, 1996).

Steroid hormones are synthesized from precursor steroid-cholesterol. Presumably, all tissues have some capacity for cholesterol synthesis, although most of the circulating cholesterol is synthesized in the liver. Adrenocortical tissue can synthesize cholesterol, and can also take it up from the blood when circulating levels are sufficiently high (Gorbman *et al.*, 1983).

In most steroidogenic tissues cholesterol that is not directly required for hormone production is stored within the cell under the form of cholesteryl esters. When a high demand for steroid hormones occurs, cholesteryl esters are cleaved to yield free cholesterol, stimulating the synthesis of steroid hormones either by making substrate available to the steroidogenic enzymes in the cell, or by increasing the transcription of several key cAMP- responsive genes that encode steroidogenic enzymes (Feige *et al.*, 1998).

There is considerable information available concerning the properties of enzymes involved in the synthesis of corticosteroids, androgens and estrogens. Most of the enzymes involved in this process belong to the family of mixed-function oxygenases known as cytochrome P450 (Bülow *et al.*, 1996).

1.3.1. - Corticosteroids

Two types of corticosteroids, mineralocorticoids (aldosterone) and glucocorticoids (cortisol, corticosterone) are produced by the adrenal cortex/ interrenal from lungfish and amphibians to mammals (figure 3). Tissue effects of glucocorticoids are mediated by the type II corticosteroid receptor- also termed the glucocorticoid receptor (GR)-, and in addition, by the mineralocorticoid receptor (MR). Cortisol has significant affinity for both receptors, while aldosterone clearly possesses higher affinity for MR (Ray, 1996).

P450 (11β) (11β-hydroxylase) or *CYP*11B (according to accepted nomenclature proposed by Nelson *et al*., 1993) catalyses the final steps of corticosteroid biosynthesis: from 11-deoxycorticosterone (DOC) to aldosterone or 11-deoxycortisol to cortisol, respectively (Jiang *et al*., 1998).

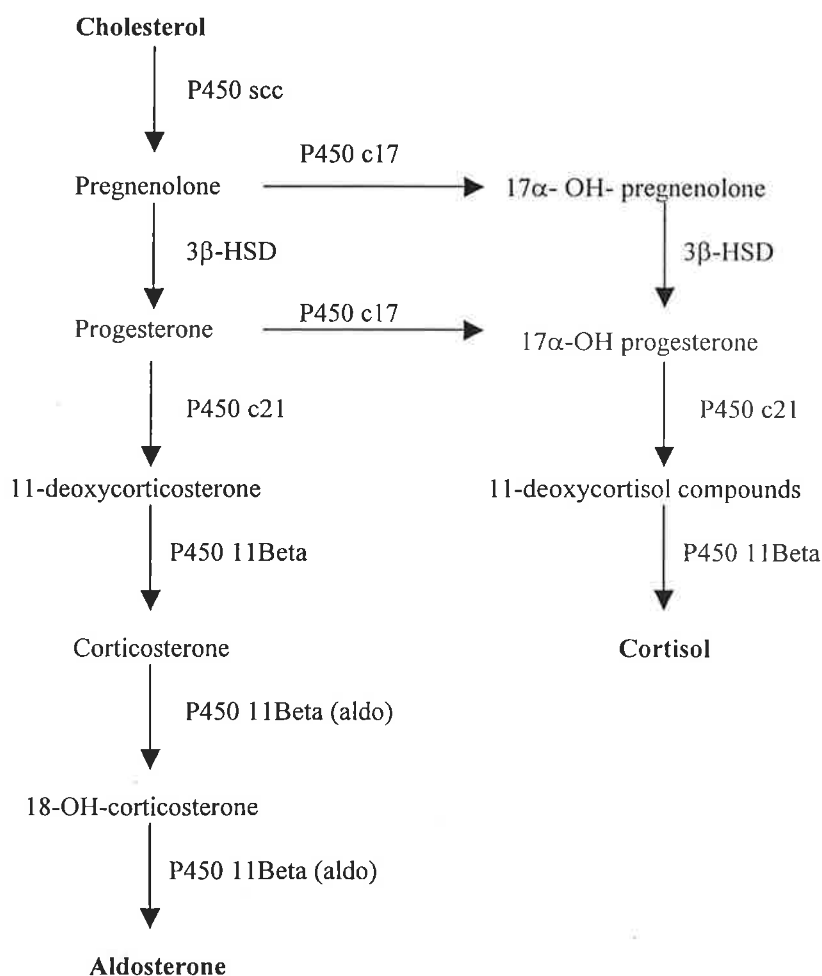


Figure 3- Schematic representation of the main pathways for corticosteroid biosynthesis (adapted from Idler, 1972).

Previous studies have shown that in several species including human (Fisher *et al.*, 2000), mouse (Domalik *et al.*, 1991), rat (Mukai *et al.*, 1993), and hamster (LeHoux *et al.*, 1994), two distinct isoforms of the *CYP*11B subfamily exist, namely *CYP*11B1 (P450 11β-hydroxylase) and *CYP*11B2 (Aldosterone synthase or AS). In bovines (Ogishima *et al.*, 1989), ovines (Sun *et al.*, 1995; Anwar *et al.*, 1998), bullfrog (Nonaka *et al.*, 1995) and Guinea pig (Büllow *et al.*, 1996), only one type of enzyme could be detected (P450 11β-hydroxylase, AS or *CYP*11B0) which has the activity of both known isozymes. In rat, in addition to these two isozymes, a third form *CYP*11B3 is also present (Mellon *et al.*, 1995).

Although some earlier studies reported that small amounts of aldosterone could be detected in the interrenal of some teleosts such as the lungfish, *Fundulus*, the coelacanth *Latimeria* and in sockeye salmon blood (Chester Jones *et al.*, 1969; Barrington, 1963), still no conclusive data has been shown for aldosterone synthesis in teleost fish.

Moreover, the *CYP* 11β (11β-hydroxylase) gene has been cloned in eel *Anguilla japonica* (Jiang *et al.*, 1998), in zebra fish *Danio rerio* (GenBank accession number BG738320), and in rainbow trout *Oncorhynchus mykiss* (Kusakabel *et al.*, 2000), but so far none of these genes present any evidence of AS activity.

1.3.2- Androgens

11-Ketotestosterone is a fish specific androgen implicated in male sexual differentiation and development (Takeo *et al.*, 1999). The enzyme P450 11β-hydroxylase is not only involved in corticosteroid biosynthesis, as it is also involved in the synthesis of 11-ketotestosterone in fish gonads and interrenal tissue. 11-Ketotestosterone is synthesized from testosterone by the actions of two enzymes, P450 11β-hydroxylase and 11β-hydroxysteroid dehydrogenase (11β-HSD) (Jiang *et al.*, 1996) (figure 4). The androgen receptor (AR) mediates most actions of 11-ketotestosterone (Todo *et al.*, 1999). So far, two different isoforms of AR (ARα and ARβ) have been identified in fish, namely in red sea bream *Pagrus major* (Touhata *et al.*, 1999), rainbow trout *Onchonrhyncus mykiss* (Takeo and Yamashita, 1999), kelp bass *Paralabrax clathratus* (Sperry and Thomas, 1999), Atlantic croaker

Micropogonias undulatus (Sperry and Thomas, 1999) and in eel *Anguilla japonica* (Ikeuchi *et al.*, 1999; Todo *et al.*, 1999).

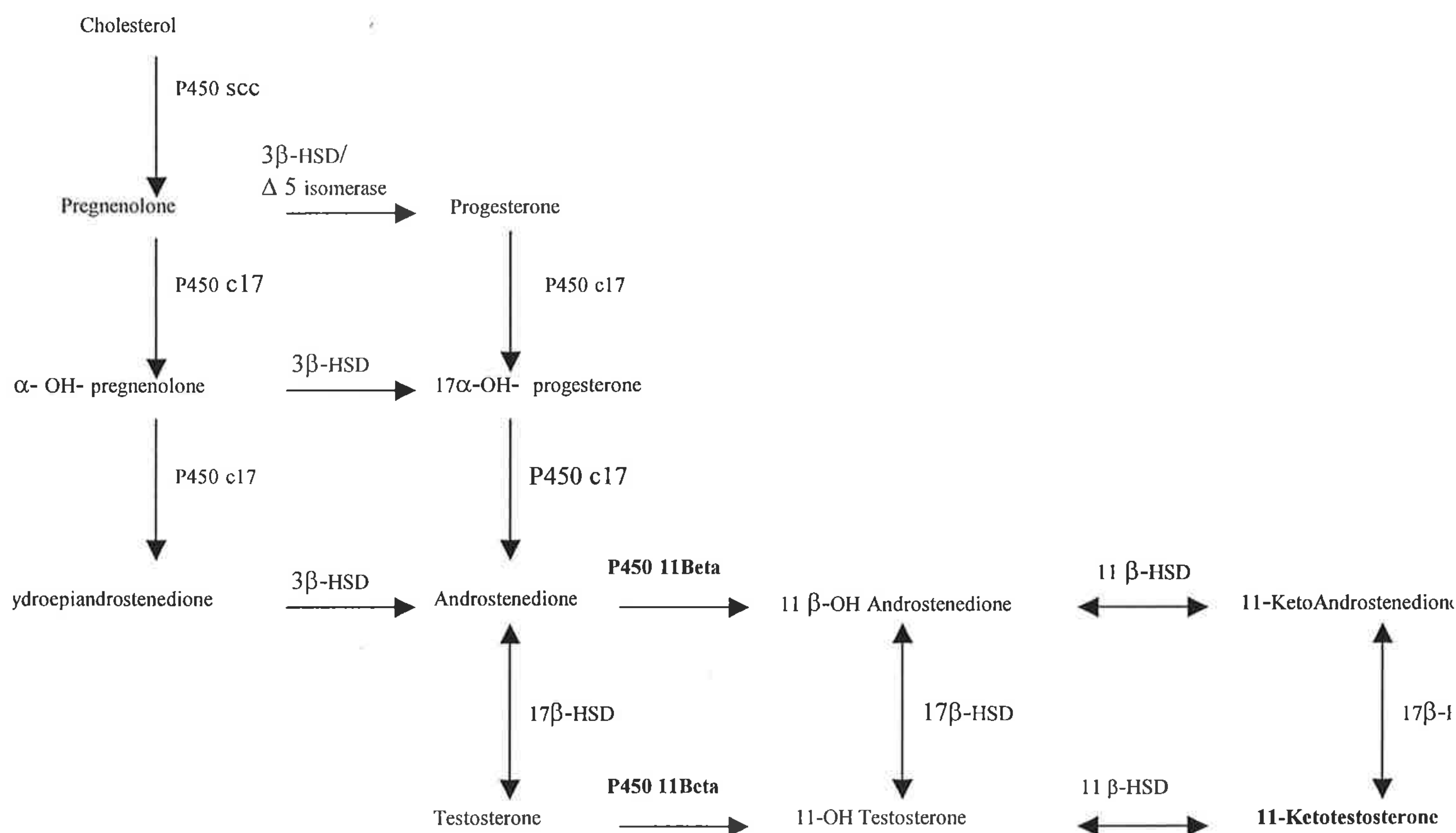


Figure 4- Schematic representation of the main pathways for androgens biosynthesis (adapted from Nagahama, 1999).

1.3.3- Estrogen

Aromatase cytochrome P450 (P450arom or *CYP*19) is the catalytic component of the aromatase complex responsible for the synthesis of estrogens from androgens. In fish it is responsible for the conversion of testosterone to 17β- Estradiol (Corbin *et al.*, 1999) and therefore plays a unique role in maintaining a physiological balance between androgens and estrogens, which is critical for gonadal development and function in vertebrates (Trant, 1994).

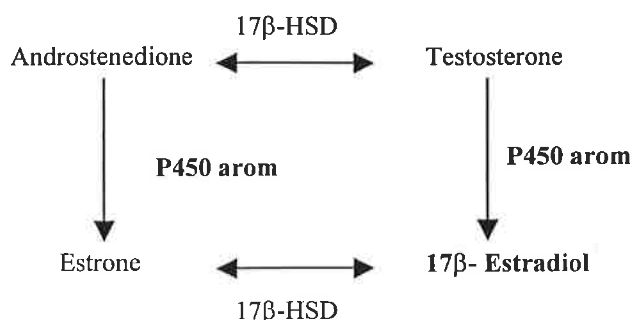


Figure 5- Schematic representation of 17β- Estradiol biosynthesis, the major estrogen in fish.

The aromatase gene has been isolated in several fish species such as tilapia *Oreochromis niloticus* (Kwon *et al.*, 2001; Chang *et al.*, 1997), Atlantic stingray *Dasyatis sabina* (Ijiri *et al.*, 2000), medaka *Oryzias latipes* (Fukada *et al.*, 1996), rainbow trout *Onchonrhyncus mykiss* (Tanaka *et al.*, 1992), zebra fish *Danio rerio* (Kishida and Callard, 2001), Atlantic croaker *Micropogonias undulatus* (Corbin *et al.*, 1999), goldfish *Carassius auratus* (Callard and Tchoudakova, 1997; Tchoudakova and Callard, 1998), Japanese flounder *Paralichthys olivaceus* (Kitano *et al.*, 1999) and sea bass *Dicentrarchus labrax* (GenBank accession number AJ311177).

In both males and females, estrogen programes and coordinates developmental, physiological, and behavioral responses essential for reproduction (Andò *et al.*, 2000; Tchoudakova and Callard, 1998). It is well established that many of the effects of estrogens are mediated by classical nuclear receptors- estrogen receptors (ER), which can bind estrogen and act as transcription factors (Kishida and Callard, 2001; Andrews *et al.*, 1997).

So far, three distinct ER subtypes (termed ERα, ERβ and ERγ) have been identified in fish, sea bream *Sparus aurata* (Socorro *et al.*, 2000), rainbow trout *Onchonrhyncus mykiss* (Pakdel *et al.*, 2000), channel catfish *Ictalurus punctatus* (Xia *et al.*, 1999; Xia *et al.*, 2000; Patiño *et al.*, 2000), Atlantic croaker *Micropogonias undulatus* (Hawkins *et al.*, 2000) and tilapia *Oreochromis niloticus* (Chang *et al.*, 1999).

II- MATERIAL AND METHODS

Experiment I: Preliminary examination of the effect of dexamethasone on plasma levels of cortisol and androgens.

Twenty sexually immature sea bass (1 year old) were distributed in two 50-liter tanks (density: 9 Kg/m^{-3}) with constant temperature (18ºC), salinity (37‰) and aeration. After one week of acclimation, the normal daily ration (commercial dry pellets, Provimi) at the rate of 1% body wt day^{-1} was replaced by a control meal or an experimental meal fed at the same rate. The pellets were prepared either by spraying food with ethanol (control) or with dexamethasone dissolved in ethanol and allowing the ethanol to evaporate overnight at room temperature. The fish in one tank were given a control meal, and the fish in the other tank received pellets with a 150 mg Kg $^{-1}$ dexamethasone (Dex). After one week of treatment, fish were allowed to recover for two weeks by feeding them with the normal daily ration of untreated pellets. Fish were sampled at the beginning of the experiment (no treatment had been administered), after one week of treatment and after one and two weeks upon treatment removal. On sampling, fish were captured, anaesthetized with phenoxyethanol (1:10000, Sigma) and blood was collected from the caudal vessels. Blood was immediately centrifuged (7 minutes at 13000 rpm), aliquoted, frozen in liquid nitrogen and stored at –20ºC, until assayed for cortisol and androgens (see below).

Experiment II: Effect of dexamethasone administration on the sex ratios of sea bass populations

Sea bass larvae 80 days-post-hatching were obtained from a fish farm (TIMAR, Peniche). Fish larvae were acclimated in 200-L tanks in a closed seawater system, equipped with biological and mechanical filters. Fish were fed twice a day with commercial food (PROVIMI) at the rate of 1-2% body wt day^{-1}. At the age of 100 days-post-hatching, 1500 larvae were distributed in six 1000-L tanks (250 larvae per tank) with constant aeration and natural ambient temperature and salinity. Four tanks were given dexamethasone treated food (two tanks with 75 mg Kg^{-1} and two tanks with 150 mg Kg $^{-1}$), prepared as previously described and other two tanks were given a control

meal. These concentrations of dexamethasone were chosen because with the previous experiment 150 mg Kg $^{-1}$ blocked cortisol production. When the fish reached 10 months of age 50 fish from each tank were anaesthetized, sacrificed and the gonads were removed for histological determination of the gonadal sex.

Experiment III: Effect of confinement and handling stress on hormone levels.

For the following experiments 2.5-year sea bass were used (mean weight 120g).

Confinement stress

Twenty sea bass were divided evenly between two 500-L tanks, ten fish per tank. After one week of acclimation, the water level in one of the tanks was reduced to half, increasing the stocking density (density: 8 Kg m^{-3}). Fish were exposed to this type of stress for 16 hours, after which they were netted, anaesthetized and blood was collected. Blood samples were treated as described above and each sample was assayed for cortisol, testosterone and 11-ketotestosterone.

Handling stress

Twenty fish from the stock population were distributed in two 1000-Liter tanks. After one week of acclimation, the fish from one tank were subjected to a handling stress. The handling stress consisted of submitting the fish to manipulation of the tank with a hand net, throughout six hours. After six hours of stress, fish were netted, anaesthetized and blood was collected. Blood samples were treated as previously described and assayed for cortisol, testosterone and 11-ketotestosterone.

Experiment IV: Effect of confinement and handling stress in sea bass treated with dexamethasone.

For the following experiments 2.5-year sea bass were used (mean weight 110g).

Confinement stress

The same confinement experiment, as previously described, was repeated but this time in one of the tanks, fish were given ethanol treated food, and the fish, which were subjected to confinement stress, were given dexamethasone treated food (150 mg Kg^{-1}). Sampling procedure was as described for experiment III and each sample was also assayed for cortisol, testosterone and 11-ketotestosterone.

Handling stress

The same handling experiment, as previously described, was repeated with some alterations. In one tank, fish were fed with ethanol treated food and the fish which were subjected to handling stress, were given dexamethasone treated food (150 mg Kg^{-1}). Sampling procedures were as previously described and the blood samples were assayed for cortisol, testosterone and 11-ketotestosterone.

2.1- *Histology*

At the end of experiment II, fish were sampled (50 fish *per* group) and the sex of each individual was determined by histological analysis. On sampling, fish were anaesthetized (1:10000 phenoxyethanol, Sigma), killed and gonads were dissected and fixed in Bouin's solution (Sigma) for 24 hours. The tissues were dehydrated in grade alcohol series, paraffin embedded and cross-sectioned (5-7μm). The cross-sections were stained with Erlich's hematoxilin-eosine and observed at the microscope. Gonads were scored as testis when they exclusively contained predominantly undifferentiated tissue with a few scattered germ cells and as ovaries when they presented groups of germ cells with solitary oocytes. Gonads that only presented undifferentiated tissue with no typical features as in testis or ovaries were classified as undifferentiated. All gonads that presented all the characteristics of the testis but also presented scattered oocytes were classified as intersexes.

2.2- *Steroid assays*

For steroids radioimmunoassay (RIA), aliquots (100µl) of plasma were denatured with (900µl) gelatine buffer at 80°C for one hour. For cortisol, the denatured plasma was diluted to 1:50 and 100µl of this dilution was used in the radioimmunoassay as described in (Condeça, 2001). The samples were counted under standard ^{3}H conditions, using a Beckman L60000IC Scintillation counter (Beckman Instruments Inc., Fullerton, USA). Testosterone and 11-ketotestosterone assays were performed using the same procedure described for cortisol assays (Condeça, 2001), with the exception that the plasma samples were not diluted prior to assay.

2.3- *RNA Isolation*

Total RNA from *Dicentrarchus labrax* tissues and whole individual larvae at different developmental stages was extracted using TRI reagent (Sigma), based in the acid guanidinium thiocyanate-phenol-chloroform method by Chomczynski and Sacchi (1987). The isolated RNA was subsequently run in a 1.0% agarose gel stained with EtBr (0.5 µg ml ¯1) to verify its integrity and quantity.

2.4- *Amplification of partial genes by RT-PCR*

Complementary DNA was synthesised from 2-4 µg of total RNA by reverse transcription in 40µl at 37°C for 2 h using 50 U of M-MLV Reverse Transcriptase (Gibco BRL), 4µl 5x Buffer (Gibco, BRL), 0.25mM of each dNTP, 20µM oligodT primer, 2.5mM DTT and 5 U of RNA Guard (Gibco, BRL). Twenty-five microliters PCR reactions were performed with 2-4µl of RT reaction in the presence of 0.5U of Taq Polymerase (Promega), 5µl 10x Buffer (Promega), 0.12 mM of each dNTP, 0.5pmol of forward and reverse primers and variable concentrations of $MgCl_2$ which varied according to the primers used for each target gene (see table 1). After PCR, the reaction products were analysed on 1% agarose gel and stained with EtBr (0.5 µg ml $^{-1}$).

forward and reverse primers and variable concentrations of $MgCl_2$ which varied according to the primers used for each target gene (see table 1). After PCR, the reaction products were analysed on 1% agarose gel and stained with EtBr (0.5 μg ml^{-1}).

Table 1: Conditions for PCR amplification of target genes.

Genes	Primers	Expected Size	PCR conditions	MgCl2 concentration	Tissue
18 S	18 S For/ 18S Rev	450 bp	94°C 30 sec 58 °C 30 sec 72°C 30 sec	1.0 mM	Undifferentiated Gonads
CYP 19	dlarom fw/ dlarom rev	400 bp	94°C 1 min 60°C 1 min 72°C 1 min	2.0 mM	Undifferentiated Gonads
CYP 11B1	3F/6R	850 bp	94°C 1 min 58 °C 1 min 72°C 1 min	2.5 mM	Kidney/HK
AR β	roar f1/ roar r1	325 bp	94°C 1 min 54°C 1 min 72°C 1 min	2.5 mM	Larvae
ER α	ER3/ER4	1000 bp	94°C 1 min 56°C 1 min 72°C 1 min	1.5 mM	Liver
ER β1	sberb1f3/ sberb1 r3	650 bp	94°C 1 min 54 °C 1 min 72°C 1 min	2.5 mM	Testis
ER β2	ER3/ER4	1000 bp	94°C 1 min 52°C 1 min 72°C 1 min	1.5 mM	Testis

2.5- *Cloning and sequencing*

PCR products with the expected size were excised from the agarose gel, and eluted from the agarose using a commercial kit (GFX™ PCR DNA and Gel band purification kit, Amersham Pharmacia), according to the manufacturer's instructions. The DNA fragments isolated were inserted into the pGem T-easy plasmid (Promega) vector and subsequently used to transform *Escherichia coli* XL-1Blue strain (Stratagene) using a standard method by Inoue *et al.* (1990). The transformed bacteria were then plated in Lb-Agar (Sigma) supplemented with 50μg/ml ampicilin (Sigma), 0.05M IPTG (Sigma) and 80μg/ml X-Gal (Sigma). The plates were incubated overnight at 37°C and the clones with the insert of the correct size were selected by PCR colony screening. For PCR colony screening, white colonies were transferred into 200 μl Lb-Broth medium (Sigma) supplemented with 50μg/ml ampicilin and incubated at 37°C for

2 hours. Two microliters of grown cultures were used in 25µl PCR reactions using 0.5U of Taq Polymerase (Promega), 2.5µl 10x Buffer (Promega), 0.12 mM of each dNTP, 0.5 pmol of T7 and SP6 primers and 2mM $MgCl_2$. The PCR conditions used were 30'' at 95°C, 30'' at 50°C and 45'' at 72°C, for 25 cycles. The colonies with inserts of the correct size were then transferred to 5ml Lb-Broth medium supplemented with 50µg/ml ampicilin and incubated at 37°C overnight. The plasmids were recovered using the alkaline lysis method (Sambrook *et al.,* 1989) and subsequently sequenced in an automated sequencer (ABI 373A) with Thermo Sequenase ™ Dye terminator cycle Sequencing Pre-Mix Kit (Amersham Pharmacia). The obtained sequences were compared to GenBank database at NCBI to determine the similarity with other known genes.

2.6- *SEMI-QUANTITATIVE PCR*

2.6.1- *PCR Optimisation*

Sea bass larvae total RNA extraction and cDNA synthesis was prepared as previously described with the exception of the primer used in the reverse transcription, which was done with 4µg of random hexamers (pdN (6), MWG- Biotech AG) in order to use the 18S as an internal control. Specific primers for the genes *CYP*11B1, *CYP*19, ARβ, ERα, ERβ1, ERβ2 and 18S were designed and PCR conditions were optimized (see table 2). For each gene, the specific primers were optimised in order to obtain a single product and the linear range for the PCR reaction was determined. To determine the linear range, a 60µl PCR reaction was set-up for each gene, 5µl aliquots were removed every two cycles, starting in cycle 16 until cycle 35 and subsequently resolved by electrophoresis on 1% agarose gel stained with EtBr (0.5 $\mu g\ ml^{-1}$). The products were quantified with the program ImageMaster 1D Primer (Pharmacia Biotech) and cycle numbers were plotted against log of the signal. Once the linear range was established, a cycle number in the middle of the linear range of the plot was chosen in order to perform semi quantitative PCR.

Table 2: Optimisation conditions for semi quantitative PCR

Genes	Primers	Expected Size	PCR conditions	MgCl2 concentration	Cycles
-	pdN (6)	-	-	-	-
CYP 19	dlaromfor1/ dlarom rev1	450 bp	94°C 1 min 58°C 1 min 72°C 1 min	2.5 mM	22
CYP 11B1	ro11b1or/ ro11b1rev	290 bp	94°C 1 min 48°C 1 min 72°C 1 min	2 mM	26
AR β	roar f1/ roar r1	300 bp	94°C 1 min 58 °C 1 min 72°C 1 min	2.5 mM	26
ER α	roera f1/ roera r1	550 bp	94°C 1 min 60°C 1 min 72°C 1 min	1.5 mM	35
ER β1	sberb1f3/ sberb1 r3	650 bp	94°C 1 min 58°C 1 min 72°C 1 min	2 mM	35
ER β2	roerb2 f1/ roerb2 r1	200 bp	94°C 1 min 48°C 1 min 72°C 1 min	2.5 mM	35
18 S	18S for/ 18S rev	450 bp	94°C 1 min 60°C 1 min 72°C 1 min	1 mM	18

2.6.2- *RT-PCR Southern Blot analysis*

PCR reactions were performed with the optimised conditions (see table 2) and subsequently run in 1% agarose gel stained with EtBr (0.5 μg ml $^{-1}$). After electrophoresis, the gel was denatured in a 1.5M NaCl/ 0.5M NaOH solution for 30 minutes, washed briefly in distilled water and neutralized in a 1.5M NaCl/ 1M Tris-HCl solution for 30 minutes. After neutralisation, the gel was washed with 6X SSC and the DNA samples were transferred into a Hybond- N Nylon membrane (Amersham Pharmacia) by a capillary method (Sambrook *et al.*, 1989). The membrane was cross-linked with UV radiation and the membrane was pre-hybridised at 58°C for 2 hours in Church-Gilbert buffer. Hybridisation was carried out overnight in Church-Gilbert buffer with the respective probe labelled with α^{32}P-dCTP and RediPrime It II Random Primer labelling kit (Statagene), according to the manufacturer's instructions. The blots were then washed twice with 2X SCC/ 0.1%SDS at RT for 10 minutes, twice with 1X SSC/ 0.1%SDS at 58°C for 20 minutes and twice with 0.1X SSC/ 01% SDS at 65°C for 30

minutes. The membrane signals were quantified by phosphoimaging and expressed as the ratio of amplified target over amplification of 18 S ribosomal RNA.

2.7- *Statistical analysis*

Results are presented as mean ± SEM. The effect of dexamethasone treatment on weight, length, condition factor, gonad differentiation and the effect of stress on steroid levels was tested by two-Way Analysis of Variance (ANOVA) followed by Tuckey's Honestly Significant Difference test. Correlation between steroid levels was assessed using the Pearson product moment correlation. Analysis of covariance (ANCOVA) was used to assess whether the effect of dexamethasone on the condition factor was due to its influence on the weight or length of the fish. Before the analysis, the data was log (weight, length, and concentrations) or inverse sine (condition factor) transformed. Plots in figures are based on untransformed data. Correlation between the expressions of the different genes was assessed using the Pearson Product Moment Correlation. Statistical significance was considered at the 5% level.

III- RESULTS

3.1- *Preliminary examination of the effect of dexamethasone on plasma levels of cortisol and androgens.*

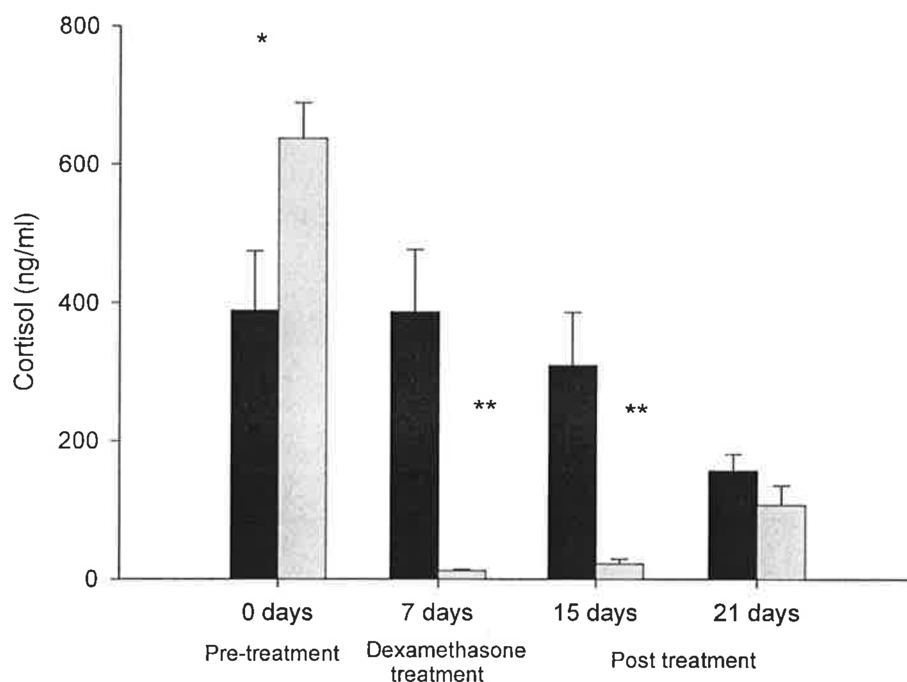


Figure 6- Dexamethasone suppression test. Black bars- control fish (n=10); Grey bars- fish treated with dexamethasone (n=10). Pre treatment- fish fed with ethanol treated food; Dexamethasone treatment- fish fed with dexamethasone at a dose of 150 mg Kg^{-1} food for one week after which they were sampled; Post treatment- Fish were sampled one week and two weeks after treatment removal. Significant differences between treatment groups are marked with * if the value of $p= 0.03$ and with ** if $p< 0.001$.

At the beginning of the experiment, the mean cortisol levels were in the range of 388.6- 636.9 ng ml^{-1} in control groups, although throughout the experiment, the levels of cortisol were significantly ($p< 0.03$) reduced (156.8 ng ml^{-1}) (fig.6). Dexamethasone at a dose of 150 mg Kg^{-1} food had a significant ($p<0.001$) suppressive effect on the plasma cortisol levels (12.13 ng ml^{-1}) after one week of steroid treatment. One week post-treatment the suppressive effect of dexamethasone was still present ($p<0.001$) and the cortisol levels only recovered to control levels, two weeks after dexamethasone

removal (fig.6). The levels of both testosterone and 11-ketotestosterone were below the detection limit of the assay.

3.2- *Effect of dexamethasone administration on the sex ratios of sea bass populations.*

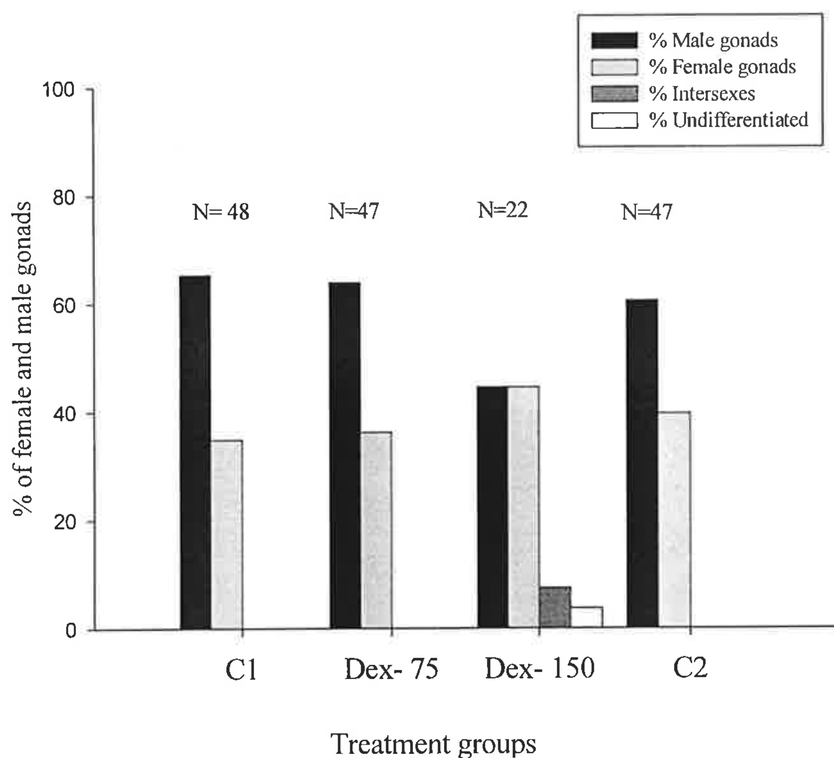


Figure 7- The effect of dexamethasone administration on the sex ratios of sea bass. C1 and C2- control fish fed with ethanol treated food; Dex-75- fish treated with 75 mg Kg^{-1} food of dexamethasone; Dex-150- fish treated with 150 mg Kg^{-1} food of dexamethasone.

Control groups had between 60-65% of males and 35-40% females; Dex-75 group presented 64% males and 36% of females and the Dex-150 group presented 44% males and 44% of males (figure 7).

When gonads were examined at 10 months of age, all fish from the control groups and those treated with the lowest dose of dexamethasone (75 mg Kg^{-1} food) presented fully differentiated gonads either into testis (figure 8.a) or ovaries (figure 8.b). 4 % of the fish treated with the highest dose of dexamethasone (150 mg Kg $^{-1}$ food) were undifferentiated (figure 8.c) and 7% had ovotestes (figure 8.d).

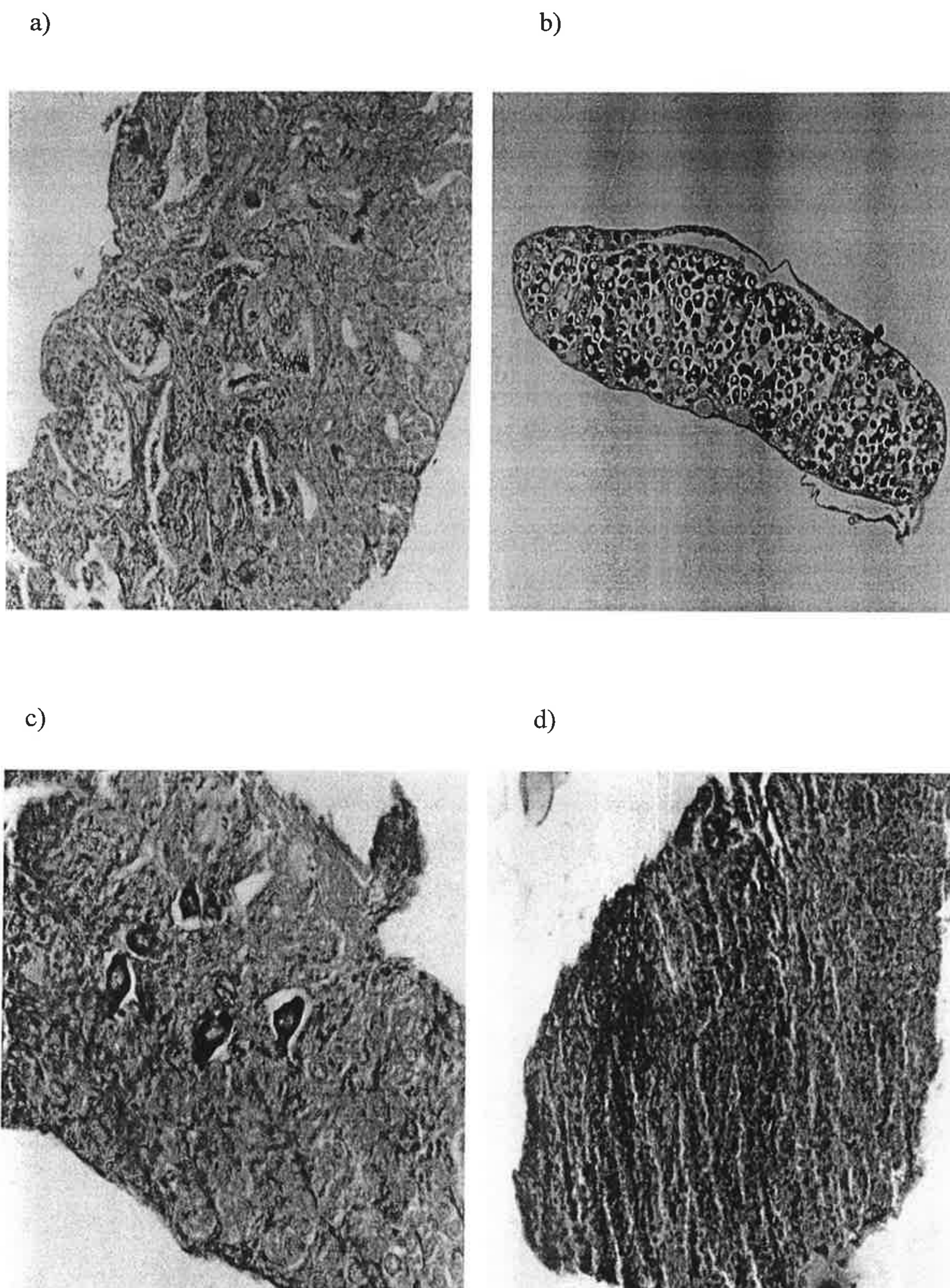
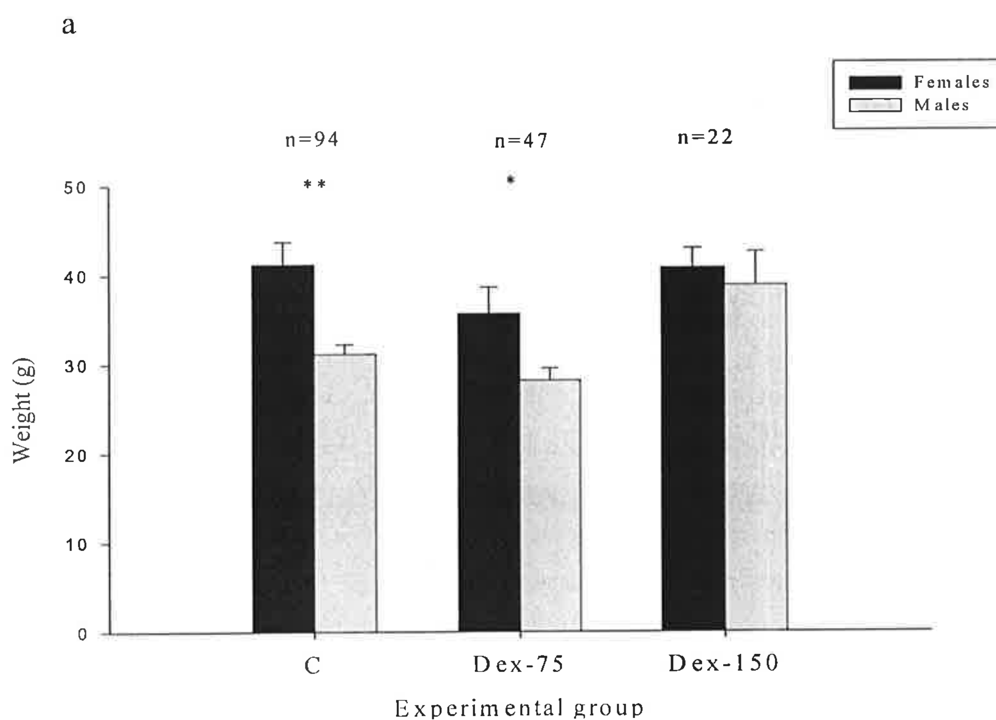


Figure 8- Histological cross sections (8μm) of gonads from experiment II at 10 months of age. a) gonad showing a developed testis; b) gonad showing a developed ovary; c) gonad showing testicular tissue with 5 intra scattered oocytes; d) undifferentiated gonad.

Since no significant differences in weight, length and condition factor could be seen between the two control groups, the data were pooled for subsequent analysis. In both control and Dex-75 groups, female fish were heavier ($p<0.001$ and $p=0.011$, respectively) (figure 9.a) and larger ($p<0.001$ and $p=0.011$, respectively) than male fish (figures 9.b). Female and male fish treated with the highest dose of dexamethasone did not present any significant differences in weight or length.

a



b

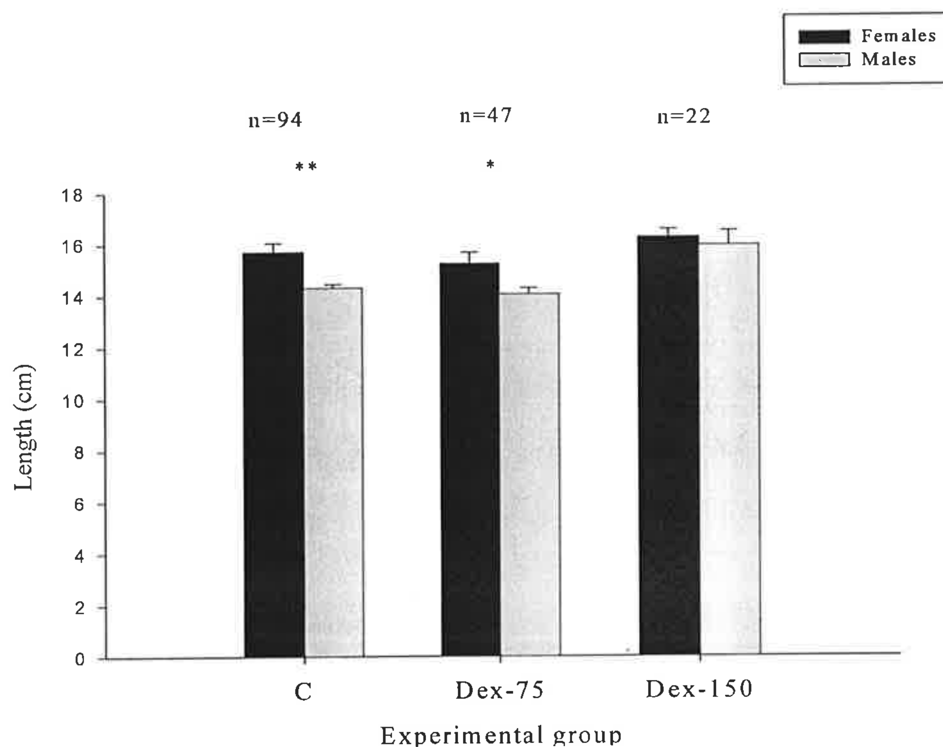


Figure 9- Fish weight (a) and length (b) at the end of the experiment II. C- control fish; Dex-75- fish treated with 75 mg Kg^{-1} food dexamethasone; Dex-150- fish treated with 150 mg Kg^{-1} food dexamethasone. Significant differences in weight and length between female and male fish in each group are marked with * if p= 0,011 and ** if p< 0,001.

There were no significant differences in condition factor between females and males within the same group. However, females from the control group had a significantly higher condition factor than fish treated with the lowest dose of dexamethasone (P = <0.001) or with the highest dose (P = <0.001) (figure 10). Similarly, male fish from the control group also had a significantly higher condition factor than males treated with the lowest (P = <0.001) and the highest (P = 0.001) dose of dexamethasone (figure 10).

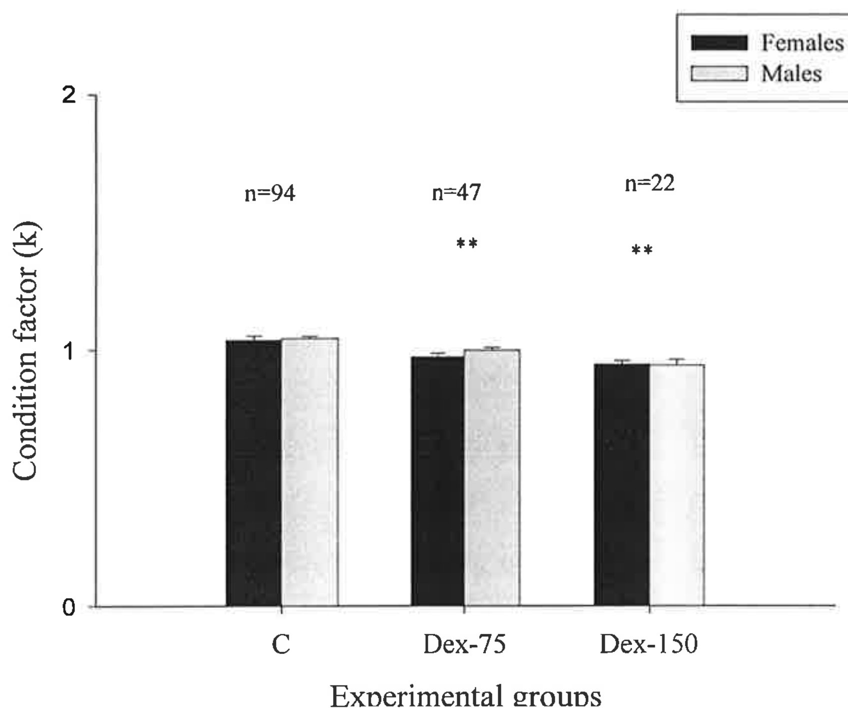


Figure 10- Condition factor at the end of experiment II: C- control fish; Dex-75- fish treated with 75 mg Kg^{-1} dexamethasone; Dex-150- fish treated with 150 mg Kg^{-1} dexamethasone. Condition factor was calculated according to the formula: $K= [(W/L^3) \times 100]$. Significant differences in condition factor in each group in comparison to the control are marked with ** if $p< 0,001$.

3.3- *Effect of confinement stress on hormone levels*

After 24 hours of confinement, there were no alterations in cortisol levels or in testosterone (figure 11). The levels of 11-ketotestosterone in most fish were below the detection limit of the assay.

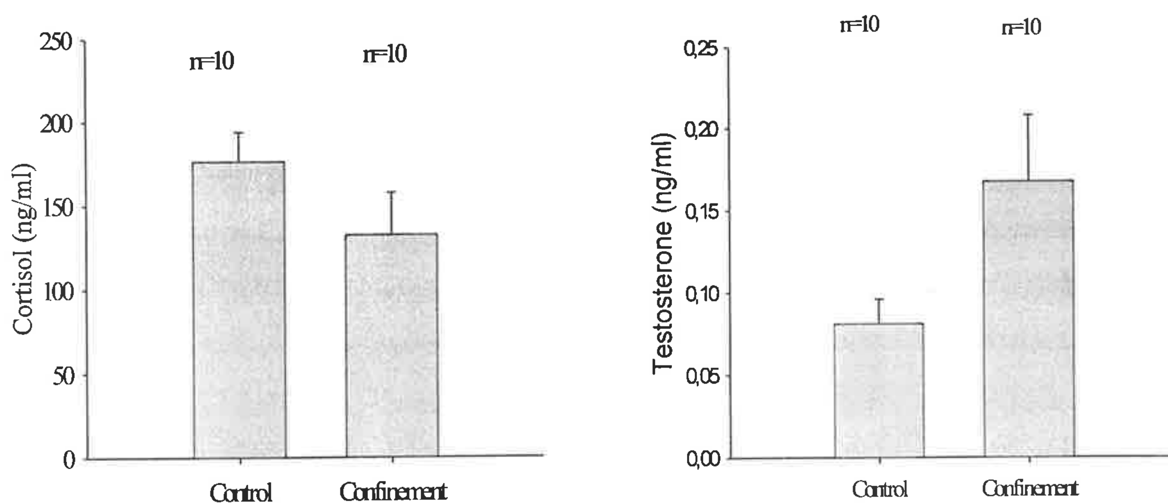


Figure 11- Cortisol and testosterone levels in sea bass blood plasma after 24 hours of confinement.

3.4- *Effect of handling stress in sea bass.*

Handling stress caused a highly significant ($p<0.001$) elevation of blood cortisol levels, and no alterations could be detected on plasma testosterone levels (figure 12). The levels of 11-ketotestosterone in most fish were below the detection limit of the assay.

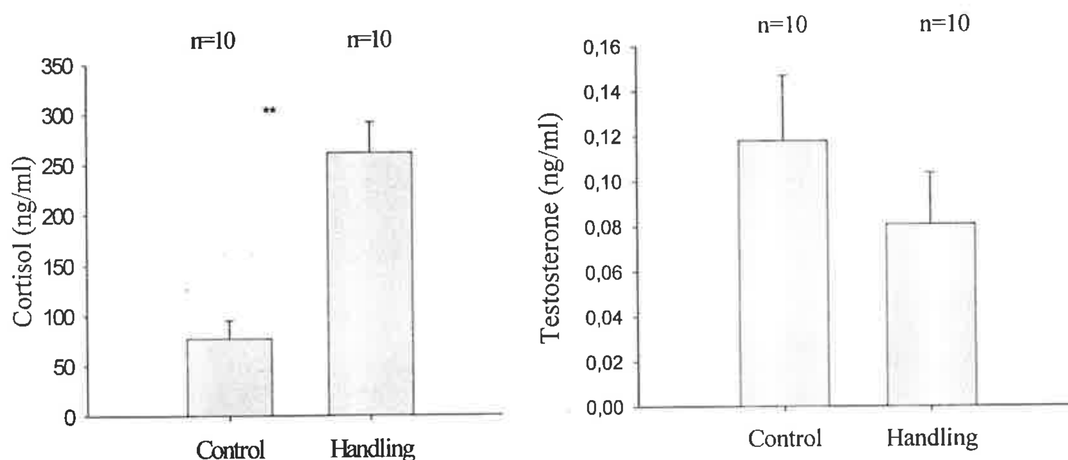


Figure 12- Cortisol and testosterone levels in sea bass blood plasma after 6 hours of handling stress. Significant differences between treatment groups are marked with ** if the value of $p< 0.001$.

3.5- *Effect of confinement stress in sea bass treated with dexamethasone.*

Dexamethasone treatment (150 mg Kg^{-1} food) had no suppressive effects on the cortisol levels after 24 hours of confinement stress (figure 13). In contrast, testosterone levels were significantly higher (p=0.01) in fish treated with dexamethasone when compared with the control fish. The levels of 11-ketotestosterone in most fish were below the detection limit of the assay.

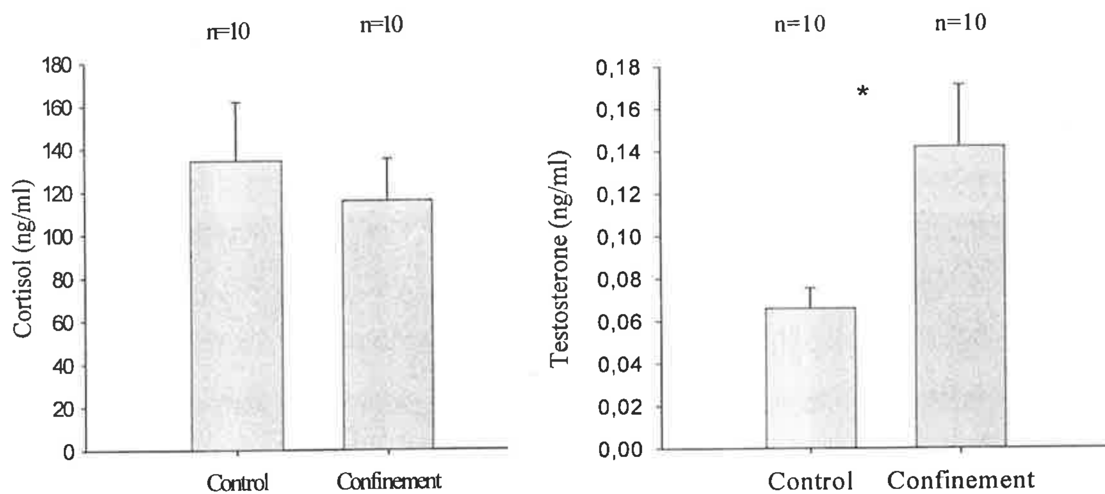


Figure 13- Cortisol and testosterone levels in sea bass blood plasma after 24 hours of confinement. Before the experiment control fish were given ethanol-treated food and fish subjected to confinement stress were given food treated with 150 mg Kg^{-1} dexamethasone. Significant differences between treatment groups are marked with * if the the value of p= 0.01

3.6- *Effect of handling stress in sea bass treated with dexamethasone.*

Handling stress caused a highly significant ($p<0.001$) increase of blood cortisol levels despite dexamethasone treatment, and no alterations on plasma testosterone levels could be detected (figure 14). The levels of 11-ketotestosterone in most fish were below the detection limit of the assay

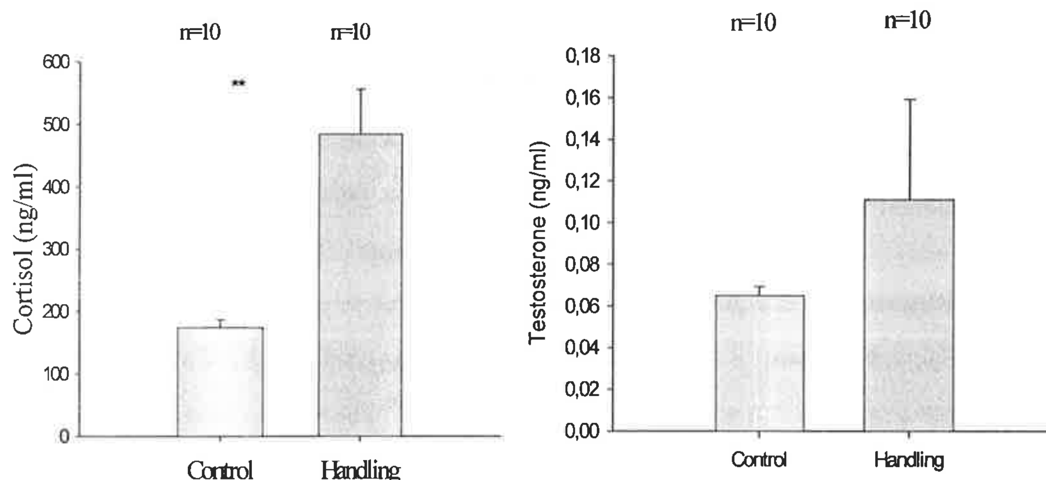


Figure 14- Cortisol and testosterone levels in sea bass blood plasma after 6 hours of handling stress. Before the experiment, control fish were given ethanol-treated food and fish subjected to handling stress were given food treated with 150 mg Kg^{-1} dexamethasone. Significant differences between treatment groups are marked with ** if the value of $p< 0.001$.

Although there appeared to be a trend for higher level of testosterone when levels of control cortisol decreased there was no significant correlation between cortisol testosterone levels for the pooled data of confinement and handling stress experiments (figure 15).

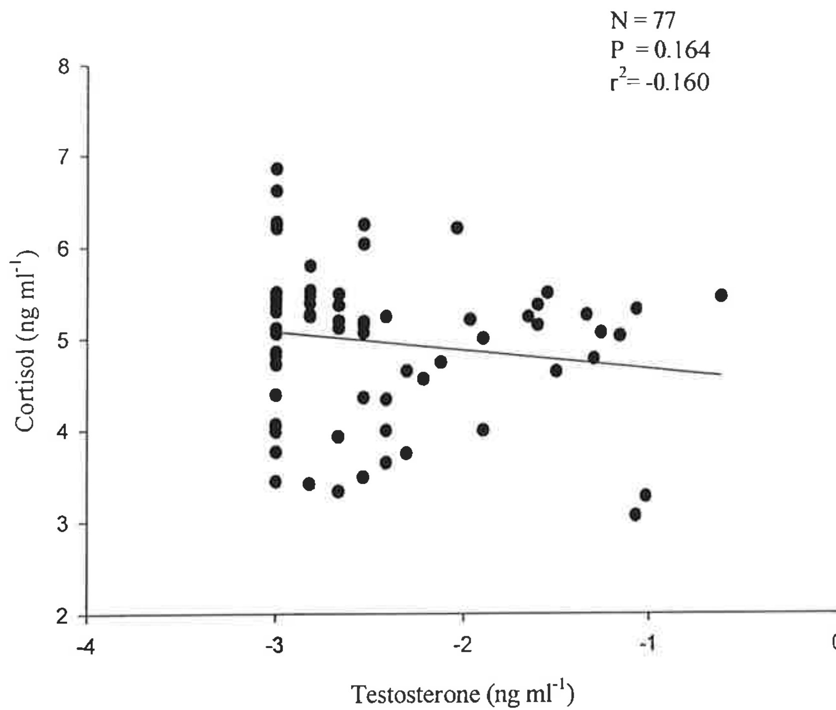


Figure 15- Correlation between cortisol and testosterone levels. Data was Ln transformed before correlation analysis was performed.

3.7- *Gene cloning*

In order to determine the enzymatic expression profile in sea bass during the period of sexual differentiation, two key enzymes involved in the final steps of estrogen and androgen synthesis were isolated- *CYP 19* and *CYP* 11B1. *CYP 19* (P450 aromatase) had 100 % similarity with the sequence that had been previously published for sea bass. As seen in table 5, the *CYP* 11B1 sequence had a high similarity to the sequence of *CYP* 11B1 isolated in *Oncorhynchus mykiss*. Four other genes, which had high similarity to the three estrogen receptors (α, β_1, β_2) identified in fish and to the androgen receptor (β), were also isolated in sea bass.

Table 5 – Target genes identified with corresponding similarity to known sequences obtained by BLAST X of Genebank.

Genes	Obtained Size	E value	Species
CYP 19	400 bp	0.0	*Dicentrarchus labrax*
CYP 11B1	850 bp	$1e^{-79}$	*Oncorhynchus mykiss*
ARβ	325 bp	$6e^{-40}$	*Pagrus major*
ERα	1000 bp	$5e^{-77}$	*Sparus aurata*
ERβ1	650 bp	$1e^{-43}$	*Sparus aurata*
ERβ2	1000 bp	$1e^{-102}$	*Micropogonias undulatus*

3.8- Gene expression- Semi-quantitative PCR

As seen in figure 16 (a), the expression of *CYP*11B1 enzyme was highest at 80 days post-hatching and showed a higher dispersion of values among individual fish. From that age onwards, the expression decreases to a stable level and the expression of this enzyme is similar in all individuals. In mature gonads, the expression of this gene is highest in testis than in the ovaries.

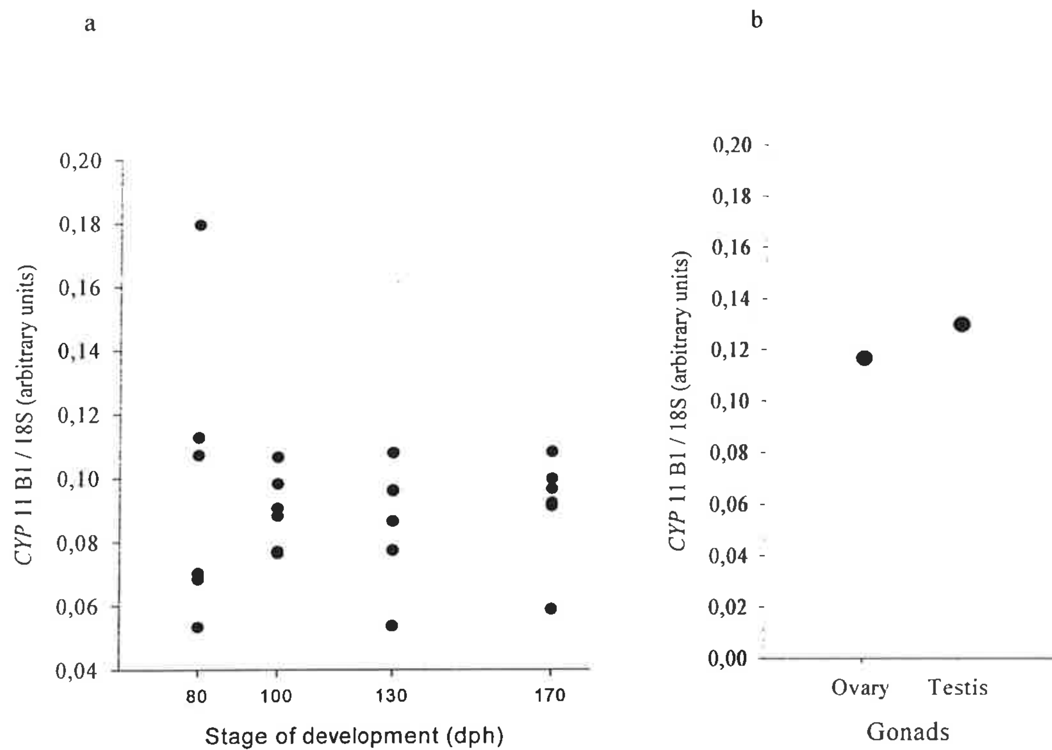


Figure 16- *CYP* 11 B1 gene expression from day 80 until day 170 post-hatching (a) and in mature gonads (b). Gene expression was measured by specific RT-PCR followed by southern blot as the ratio of amplified target over amplification of 18S ribosomal RNA.

The highest expression of androgen receptor was observed at 80 days post-hatching, and after it progressively decreased (figure 17 a). From day 80 until day 130 post-hatching, the expression of this gene presented a dimorphic pattern. The expression of this gene was higher in testis than in the ovaries (figure 17 b).

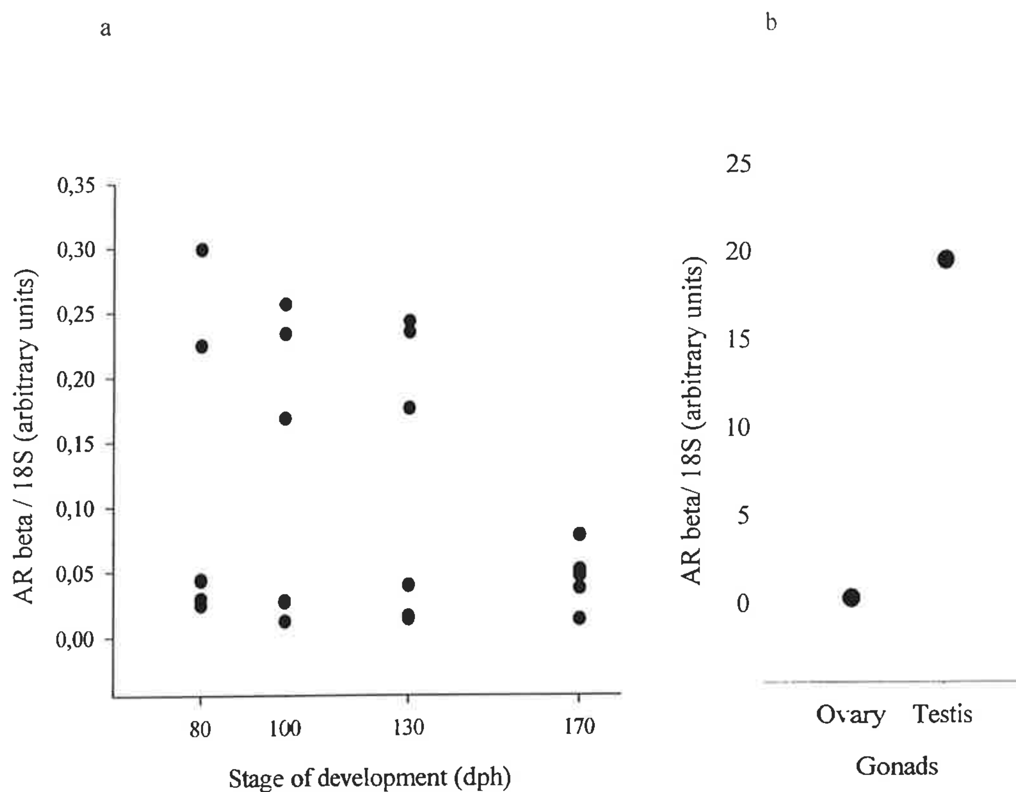


Figure 17- AR beta gene expression from day 80 until day 170 post hatching (a) and in mature gonads (b). Gene expression was measured by specific RT-PCR followed by southern blot as the ratio of amplified target over amplification of 18S ribosomal RNA.

As seen in figure 18 (a), the enzyme *CYP* 19 showed low expression levels from day 80 to day 130 post-hatching, after which it increased and showed a high dispersion of values among the different individuals. In mature gonads, this gene had higher expression in ovaries than in testis (figure 18 b).

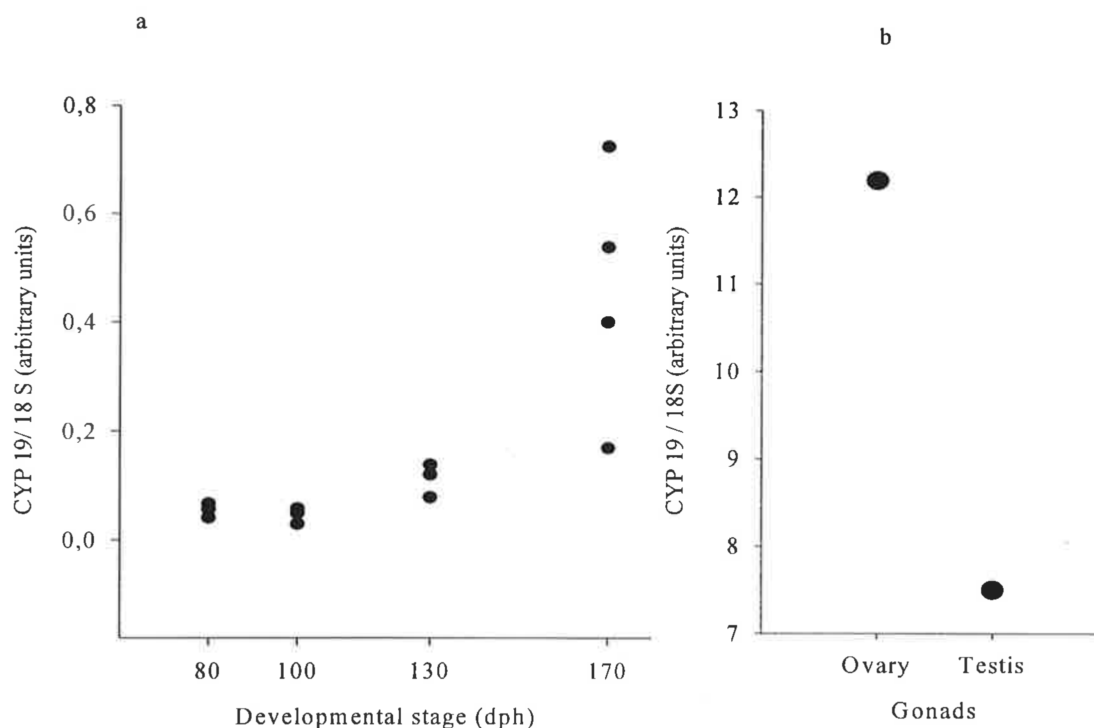


Figure 18- *CYP* 19 gene expression from day 80 until day 170 post hatching (a) and in mature gonads (b). Gene expression was measured by specific RT-PCR followed by southern blot as the ratio of amplified target over amplification of 18S ribosomal RNA.

The expression of estrogen receptor subtype α was not detected at any age or in the gonads.

The expression of the estrogen receptor subtype β1 was similar at all ages, even though it showed a slight and progressive dispersion of values from day 80 to day 130 post-hatching (figure 19 a). The expression of this receptor was higher in ovaries than in testis (figure 19 b).

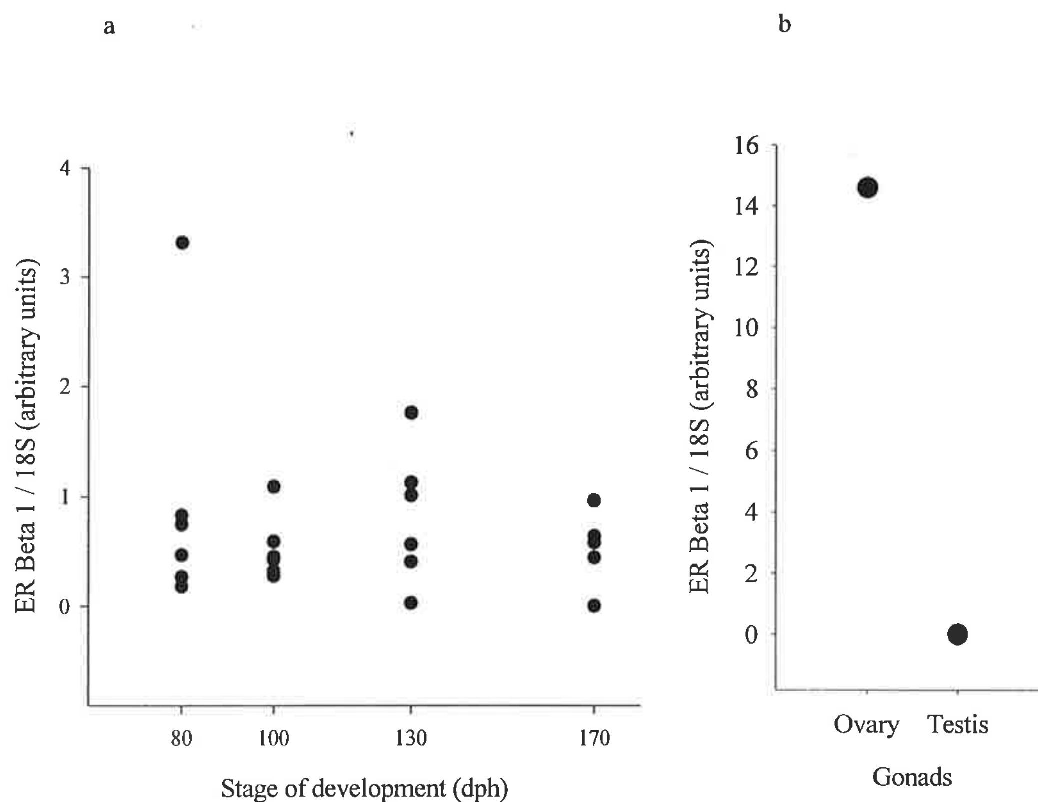


Figure 19- Estrogen receptor β1 gene expression from day 80 until day 170 post-hatching (a) and in mature gonads (b). Gene expression was measured by specific RT-PCR followed by southern blot as the ratio of amplified target over amplification of 18S ribosomal RNA.

The estrogen receptor subtype β2 had low expression levels from day 80 to day 100 post-hatching (figure 20 a). From day 130 onwards, the expression of this gene increased and showed a high dispersion of values among the different individuals. In mature gonads, this gene showed higher expression in testis than in ovaries (figure 20 b).

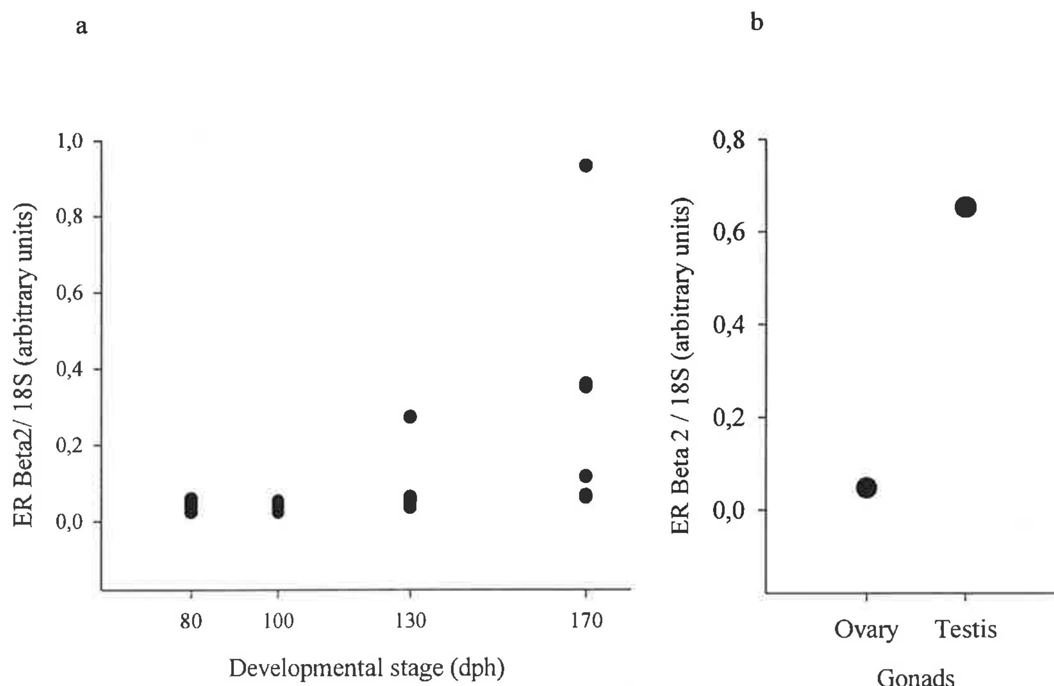


Figure 20- Estrogen receptor β2 gene expression from day 80 until day 170 post-hatching (a) and in mature gonads (b). Gene expression was measured by specific RT-PCR followed by southern blot as the ratio of amplified target over amplification of 18S ribosomal RNA.

Altogether, the analysis of these results showed that, sea bass larvae had higher expression of *CYP* 11B1 and ARβ during the androgen sensitive period. After this period, the expression of these genes was inversely correlated to the expression of the ERβ2 (table 6).

Table 6- Correlation between the expression of AR, ERβ2, and *CYP* 11B1 from day 130 until day 170 post hatching.

Cell Contents:
Correlation Coefficient
P Value

	ARβ	ERβ2
CYP 11B1	0.650 0.162	-0.820 0.0422
ARβ		-0.853 0.0310
ERβ2		

IV- DISCUSSION

Valuable information has been obtained by the administration of hormones and drugs to modify the activity at various points along the HPI axis. Dexamethasone, a synthetic glucocorticoid, has been shown to be effective in suppressing the axis both in mammals (Brody *et al.*, 1994; Swab *et al.*, 1989; Forest and David, 1992) and in fish (Pickering *et al.*, 1987; Lee *et al.*, 1992; Pottinger *et al.*, 2000). This synthetic glucocorticoid has been shown to inhibit corticotrophin-releasing hormone (CRH) in the hypothalamus, and specifically inhibit proopiomelanocortin peptide (POMC) secretion from the anterior lobe, and by thus suppress the secretion of α-MSH and ACTH in the pituitary (Kooistra *et al.*, 1997). As a result, the cortisol production in the interrenal tissue is suppressed. Furthermore, dexamethasone has also been shown to bind to glucocorticoid receptors (Knoebl *et al.*, 1996) with higher binding affinity than endogenous cortisol (Allison and Omeljaniuk, 1998). For all these reasons, dexamethasone was tested for its effectiveness in suppressing the HPI axis in sea bass.

One week of dexamethasone administration at a dose of 150 mg Kg^{-1} food effectively suppressed the cortisol secretion. The levels of cortisol were only restored to control levels after two weeks upon removal of the treatment. In brown trout, one single dose of dexamethasone (200 mg Kg^{-1}) had a marked suppressive effect on the cortisol response to confinement stress (Pickering *et al.*, 1987). However, the suppressive effect had disappeared 96 hours post treatment. These contrasting results between the two studies are not surprising since differences in frequency of administration and length of the treatment, together with the prolonged plasma half-life of this glucocorticoid (Brody *et al.*, 1995), have been shown to delay the recovery of the axis.

Determining basal levels of cortisol is one of the most common problems in most studies, as factors such as the method of capture and blood sampling, season, time of the day and sex can all influence plasma cortisol levels (Planas *et al.*, 1990; Haddy and Pankhurst, 1999). In this experiment it is noteworthy the extremely elevated cortisol levels before administration of the treatment. However, considering that throughout the experiment, the subsequent blood analysis of untreated fish presented consistently lower cortisol levels, it is likely that the initial levels were due to the stress induced by capture prior to first blood sampling.

Overall, dexamethasone was shown to be effective in suppressing the HPI axis in sea bass. Since in most fish species, embryos/larvae are able to synthesise and

metabolise cortisol (Barry *et al.*, 1995), and the HPI axis can be activated in response to stress (Stouthart *et al.*, 1998), then it is most likely that the HPI axis in larvae is also susceptible to dexamethasone inhibition. If dexamethasone is effective in suppressing the HPI axis during the period of highest sensitivity to androgens, both cortisol and as hypothesised the androgen levels should decrease, and thus invert the skewed sex ratios in favour of females.

For effective sex reversal, steroids must be administered at a sufficient dosage while the gonad is undifferentiated, and must continue to be administered throughout the period of sexual differentiation (Hendry *et al.*, 1999). For this reason, sea bass larvae were given dexamethasone throughout most of the period of sexual differentiation, starting at the beginning of the androgen sensitive period (100 DPF), when the gonads are still undifferentiated, and ending when gonads were fully differentiated (10 months).

At the end of the experiment, untreated fish (controls) had skewed sex ratios in favour of males (60-65% males), similar to most sea bass cultured stocks so far reported. Treatment with 75 mg Kg^{-1} food dexamethasone did not alter the sex ratios in sea bass. Treatment with 150 mg Kg^{-1} food dexamethasone lead to a population with a sex ratio of 1.00, but the latter result could be due to selective mortality, since only a limited number of fish survived the experiment (n=22). Despite the high mortality, it is important to notice that in this treatment, 4% of the fish were still undifferentiated at 10 months of age and 7% of the fish presented testicular tissue with scattered intra testicular oocytes (ovotestes).

Steroid administration has been reported to induce the appearance of intersexes in reptiles (Dorizzi *et al.* (1994), and fish (Blázquez *et al.*, 1995). In sea bass it has been reported the existence of testicular tissue with scattered intra testicular oocytes (ovotestes) when fish exposed to 17α-methyl testosterone (Blázquez *et al.*, 1995; Chatain *et al.*, 1999). However, in these same studies, it was also reported that fish, which had not been exposed to any hormonal treatment, also presented some cases of intersexes. Similar results have also been reported by Strüssmann *et al.* (1996) in pejerrey (*Odontesthes bonaiensis*) during the process of gonad differentiation. Even though intersexes may appear in fish that were not exposed to any exogenous steroids, in our study, the cases of intersexes were exclusively present in the group exposed to the highest dose of dexamethasone. This suggests that dexamethasone may have had an effect in tissue differentiation, but probably it only induced a temporal shift of gonad

differentiation in the female direction. Consequently, normal testicular development could have been resumed when the transformed gonads failed to produce their own feminising steroids. In fact, it has been shown that excessive treatments can lead to disruptions in gonadal development, which, in most cases, reflect incompatibilities between the exogenous and physiological processes, or pathological effects on gonadal development (Devlin and Nagahama, 2002). In this respect, species differences point out for a contradictory effect of dexamethasone on testicular activity. For example, in several vertebrates, e.g. reptiles and mammals, adrenalectomy caused a marked suppression of the steroidogenic activity of the testis. However, administration of dexamethasone resulted in normalization of testicular activity (Yajurvedi and Chandramohan, 1994). In contrast, dexamethasone has been shown to inhibit testicular steroidogenesis in rats when administered at high doses or for extended periods (Gow *et al.*, 2001). Thus, it is possible that the extended period of administration of dexamethasone in this work may have been the reason for the observed intersexes in sea bass, rather than a direct effect of corticosteroids on gonadal differentiation.

This experiment was performed during the period of highest sensitivity to androgens, but the period in which these fish exhibit increased sensitivity to exogenous steroid treatments starts earlier, around 57 DPF (Pavlidis *et al.*, 2000). Thus, the timing at which dexamethasone was administered in this experiment may have been one of the factors that could account for the failure to alter the sex ratios. In fact, earlier studies on sexual differentiation in sea bass have shown that the time frame at which a treatment is administered is determinant. For example, two different studies have reported different effects of the same range of temperatures on the sex ratios in sea bass, depending on the stage of development at which the experiment was performed (Pavlidis *et al.*, 2000; Blázquez *et al.*, 1995), and a third experiment suggest that sex liability may be genetically determined (Saillant *et al.*, 2002).

During gonad development, sea bass males divert energy resources into gonad development, while females use most resources for somatic growth. Thus, females grow faster than males (Blázquez *et al.*, 1995). Similarly, in this experiment, both in control fish and Dex-75 treated fish, females were heavier and larger than males. Comparing the weight and length of either female or male fish from these two groups, it is clear that fish that received dexamethasone treatment had significantly less weight and were smaller than fish from the control group. These results are in accordance to several reports in humans (Brody *et al.*, 1994), where glucocorticoid treatment has been shown

to decrease the weight and length, when administered at high doses and throughout extended periods. In fact, glucocorticoids have been shown to affect protein, carbohydrate and lipid metabolisms, and also to be directly involved in regulating the transcription of both growth hormone (GH) gene (in rats) (Jux *et al.*, 1998) and growth hormone receptor (GHR) in fish (Bernardini *et al.*, 1999).

In most fish species, length is positively correlated with weight, a relationship that can be expressed by the condition factor. Thus, the condition factor will remain constant no matter how large a fish becomes providing that linear proportions (shape) remain constant, which for practical purposes can be applied for sized fish, as is the case in these experiments. A change of weight at a particular length, or a change of length, without corresponding change in weight, will alter the condition factor. Fish populations display changes in average condition over time, reflecting fluctuations in their metabolic balance and also during sexual development (Weatherley and Gill, 1989). In this experiment, there were no significant differences in condition factor between female and male fish within each experimental group. However, there were significant differences in condition factor between control fish and fish from both Dex-75 and Dex-150 experimental groups, so that administration of dexamethasone decreased the condition factor of the fish, in a dose dependent manner.

Contrary to all other fish groups, there were no significant differences in weight and length between females and males in Dex-150 treated fish. It is important, however, to notice that due to high mortality, the Dex-150 fish were grown with lower density than the rest of the fish groups, and it is also known that population density influences the growth rate of fish (Weatherley and Gill, 1989). Thus, in this experiment, the different density in the Dex-150 treated fish may have been great enough to allow both female and male fish to grow as much as control fish.

Earlier studies on sexual differentiation in sea bass have shown that the size of the fish is a critical marker of the timing of sex differentiation (Blázquez *et al.*, 1998), even though it is still not known whether within a certain population, individuals become males because of their smaller growth, or smaller because of their maleness (Pavlidis *et al.*, 2000). Thus, if size is indeed determinant for sexual differentiation then, it is not possible to determine if the doses of dexamethasone that were administered had no effect on sexual differentiation, or if the effect of this steroid on growth was great enough to overcome its effect on gonad development. In fact, the presence of intersexed fish was only observed in treated fish grown under low density (Dex-150) and was also

where the fish were bigger than untreated fish, despite dexamethasone treatment. Thus, it is possible that the lower density at which the fish were raised may have counter balanced the influence of dexamethasone on growth in these fish, and thereby the effect of dexamethasone on gonad development could be detected.

Altogether, it should be taken into account that glucocorticoids have been shown to function both as inhibitors and stimulators of GH secretion and/or action, in a time- and dose-dependent manner (Giustina and Veldhuis, 1998). Therefore, it is possible that there is a threshold value, above which, the growth and metabolism of the fish is affected, which may have been the case of the doses administered in sea bass throughout this study.

Regardless the effects of dexamethasone on growth, there was no conclusive evidence demonstrating a role for the interrenal on sexual differentiation. It is possible that in sea bass, in contrast to salmonid species, the elevated stress responses are not accompanied by the increased release of androgens. To test this hypothesis two stress experiments were designed, and both cortisol and androgens were measured in fish that were exposed to either chronic (continuous) or acute (short-term) forms of stress.

In the confinement stress experiment, the levels of cortisol in unstressed sea bass were of a similar magnitude to other fish species, e.g. common carp (Pottinger *et al.*, 1999), goldfish (Pottinger *et al.*, 2000), chub (Pottinger *et al.*, 2000), and brown trout (Pottinger *et al.*, 1996). Plasma cortisol levels of chronically confined sea bass were similar to control levels, showing no signs of a response to the stressor. These results are in agreement with earlier reports where confinement stress did not also elicit a stress response in other fish species, e.g. juvenile pallid sturgeon (*Scaphirhynchus albus*) (Barton *et al.*, 2000), red porgy (Rotllant and Tort, 1997), and rainbow trout (Pottinger *et al.*, 1996).

Despite the well-documented increases in plasma cortisol in response to different types of stress, not all stressful stimuli will necessarily result in chronic elevations of cortisol levels (Rotllant *et al.*, 2000). For example, it has been shown that several factors such as individual variation, strain of fish and repetition of the stimulus may modify the HPI axis response to stress (Pottinger *et al.*, 1996). In this experiment, confinement did not induce any alteration to the levels of cortisol in sea bass. However, even though the levels of cortisol observed in unstressed sea bass were within the range of some other species, they still exceeded the levels of cortisol reported to elicit adverse effects on growth, reproduction, and in the immune system of, for example, salmonid

species (>80 ng ml^{-1}). Thus, the failure to induce a stress response in sea bass may have been due to the fact that they already possessed a higher set point regulation for glucocorticoids, and therefore reduced sensitivity for stressors. Moreover, it has been shown that there are strain differences in cortisol responsiveness to stressors, and that the relative magnitude of the plasma cortisol response to stress of individual fish is a stable trait within a proportion of the population (Pottinger and Carrick, 1999; Overli *et al.*, 2002; Pottinger and Carrick, 2001). In salmonids, the demonstrated heritability of the stress response (Fevolden *et al.*, 1993), suggest that domestication pushes populations through a narrow selection gate for low sensitivity to stress (Pankhurst and van der Kraak, 2000). Thus, it is likely that the fish used in this experiment already presented less sensitivity to stress, which could explain why no response was observed after they had been subjected to overcrowding.

Another possibility is that the fish are quick to adapt to the continuous presence of a stressor. In tilapia (*Oreochromis mossambicus*), high responses to several stressors have been reported (Balm *et al.*, 1994). However, it has also been shown that the animals are able to rapidly lower the plasma cortisol levels in the continuous presence of the stressor, which is facilitated by the short half-life of cortisol in tilapia plasma (Foo and Lam, 1993). The ability to quickly down regulate the activity of the HPI axis is advantageous to fish since in this way they can avoid the deleterious consequences of prolonged elevations in plasma cortisol. Thus, low cortisol response to high stocking density could be indicative of enhanced cortisol clearance, as mixed-function oxygenated and conjugating enzymes may stimulate inactivation and excretion of corticosteroids (Rotllant *et al.*, 2000). This leads to the assumption that depending on the time frame studied, stress related elevations in circulating cortisol could be misinterpreted and that if these fish had been exposed for a different period of time to the stressor, a response to the stressor could have been detected.

In addition, the level of cortisol in the blood of both unstressed and stressed fish is also subject to modulation by a number of internal factors, including the degree of sexual maturation (Pottinger *et al.*, 2000; Planas *et al.*, 1990). For example, several salmonid species appear to be less sensitive to stress at more advanced stages of sexual maturity (Pankhurst and van der Kraak, 2000); e.g. in brown trout, mature male fish respond to a standardized stressor with a significantly lower elevation of blood cortisol than in immature fish (Pottinger and Carrick, 2000), and it has been suggested that the reduced cortisol response to chronic stress is the result of elevated androgens present

during sexual maturation (Pottinger *et al.*, 1996). In the present work, however, both testosterone and 11-ketotestosterone were present only at very low levels and therefore the low response to confinement stress in sea bass cannot be related to the levels of androgens in blood plasma.

Even though 24 hours of confinement did not elicit a stress response in sea bass, short-term stress (handling) did induce a highly significant elevation of cortisol in blood plasma. Similar results have been reported in different fish species, e.g. gilthead sea bream (Rotllant *et al.*, 2001), rainbow trout (Blom *et al.*, 2000; Demers and Bayne, 1997, Pickering *et al.*, 1991), Chinook salmon (Palmisano *et al.*, 2000), Coho salmon (Shrimpton and Randall, 1994) and whitefish *Coregonus lavaretus* (Lappivaara, 2001).

Despite the elevation of cortisol levels in response to acute stress, no significant alterations were observed in androgen levels. Even though enhanced testosterone secretion in response to certain stressors was detected in some studies, in other studies either no effect or a marked suppression of androgens in blood plasma was found. In brown trout and rainbow trout (Pickering *et al.*, 1987), acute handling stress induced a marked suppression of testosterone and 11-ketotestosterone levels in blood plasma. However, this effect is not always consistent within the same species since studies among different salmonid species (Pottinger *et al.*, 1995; Young *et al.*, 1996) and non-salmonids (Hobby *et al.*, 2000; Campbell *et al.*, 1994) failed to show a consistent suppression of androgens by stress. Several hypotheses have been given to justify these inconsistent data. For example, Pankhurst and van der Kraak (2000) suggested that plasma cortisol needed to reach a certain absolute threshold before depression of plasma testosterone could occur, and Fenske (1997) proposed that the inhibition of testosterone takes place if the intracellular cortisol concentration exceeds the capacity of the 11β-HSD to inactivate it (Fenske, 1997). Furthermore, it has been shown that in some vertebrate species, the testosterone response to stress depends on the type of stressor applied. In adult white Leghorn cockerels, confinement stress increased the cortisol levels in blood plasma, but no significant alteration was observed in testosterone levels. However, the same regime of blood sampling applied to animals that had not been exposed to any type of stress, led to a significant elevation of testosterone levels (Heiblum *et al.*, 2000). Altogether, it is possible that: (1) in sea bass there is no correlation between the testosterone and cortisol levels during stress; or (2) the levels of testosterone are simply not affected by the specific type of stressor to which they were

subjected; or (3) that the magnitude of the stressor was not great enough to influence the production and/or release of testosterone.

Together with the stress experiments that were discussed above, two other stress experiments were simultaneously carried out, differing only in the fact that the fish that were exposed to either confinement or handling stress had been treated with dexamethasone for one week until the blood was sampled. Dexamethasone treated sea bass exposed to confinement stress had approximately the same cortisol levels as untreated fish. Considering that dexamethasone had been previously shown to be effective in suppressing the cortisol levels in sea bass, then if these fish did also not react to confinement, it would be expected that at least the levels of cortisol would be lower than untreated fish, which suggests that dexamethasone or the dose administered may have not been suppressing the HPI axis of these fish. Moreover, since in most fish species the level of cortisol in the blood of both unstressed and stressed fish is subject to modulation by the degree of sexual maturation (Pottinger *et al.*, 2000; Planas *et al.*, 1990), then it is possible that there may be also a different sensitivity to dexamethasone with the increase of age, or that mature fish have a different set point regulation for glucocorticoid levels and therefore the dose of dexamethasone was probably no longer effective in mature fish. It is possible, however, that confinement did indeed elicit a stress response in these fish and that the doses of dexamethasone administered were enough to block the stress response, but not enough to decrease basal production.

Similarly, the levels of testosterone in fish treated with dexamethasone were also within the same range as those in untreated fish.

Acute stress on the other hand, induced a highly significant response as shown by the elevated cortisol levels, despite dexamethasone treatment. These results suggest that once again dexamethasone administration did not appear to have any suppression effect of the HPI.

In teleosts, corticosteroids secreted by the interrenal tissue exert a classical or a long negative feedback loop at the level of the hypothalamus and/or pituitary to influence ACTH secretion (Bradford *et al.*, 1992). However, several studies have provided evidence for the involvement of multiple hypothalamic (corticotrophin releasing hormone- CRH and thyroid releasing hormone- THR) and pituitary (ACTH and melanocortin stimulating hormone- α- MSH) peptides on the activation of the HPI axis triggered by stressful conditions in fish (Rotllant *et al.*, 2000; Rotllant *et al.*, 2000 b). These studies point out that species differences in the cortisol response to a stressor

reflect different responses of not only the corticotropes (ACTH) but also of the melanotropes of the pituitary gland (Rotllant *et al.*, 2000; Rotllant *et al.*, 2000 b; Ruane *et al.*, 1999). Several studies have reported that in some vertebrates (e.g. dogs) dexamethasone inhibits CRH, and specifically inhibits proopiomelanocortin peptide (POMC) secretion from the anterior lobe and thus suppresses the secretion of α-MSH and ACTH in the pituitary (Kooistra *et al.*, 1997). However, species differences exist. For example, in rats after dexamethasone pre treatment, ACTH release is blocked but α-MSH release is not affected (Szalay and Folly, 1992; Katalin *et al.*, 1992). In addition, the production of α-MSH in response to a stressor has been shown to be capable of inducing the production of cortisol in some fish species (e.g. sea bream) (Rotllant *et al.*, 2000). Altogether, this suggests that even though the production of ACTH in sea bass may have been reduced by dexamethasone, the α-MSH release may have not been affected and thereby able to stimulate cortisol production in response to acute stress, regardless dexamethasone administration.

In some vertebrate species, the regulation of the stress response is not always linked to the HP axis. Studies with lines of rainbow trout selected for high (HR) and low (LR) responsiveness to a standardised stressor have shown that there is a sustained divergence in plasma cortisol levels between the two lines of fish, although no significant differences in plasma ACTH levels were evident. In fact, the plasma cortisol levels in DEX-blocked HR and LR fish after sham injection were low, but also significantly different between the two groups. These results indicate that modulation of cortisol responsiveness to stressors in HR and LR fish resides, at least in part, downstream of the hypothalamic-pituitary axis (Pottinger and Carrick 2001 b). In fact, studies with sea bream (*Sparus aurata*) have shown that confinement induced a cortisol response without changes in ACTH levels, and also provided evidence that a stressor-specific activation of the brain-sympathetic-chromaffin cell (BSC) and brain-pituitary-interrenal (BPI) axes may occur in fish (Arends *et al.*, 1999). In trout (*Salmo gairdnerii*), the production and/or release cortisol is also under the influence of other hormonal substances acting on the adenyl-cyclase system (Gupta *et al.*, 1985), independent of ACTH regulation. In salmonids, it has also been shown that cortisol may it self exert an ultra-short-loop negative feedback at the level of the interrenal tissue, functioning as a degree of self-regulation, which does not involve the H-P axis (Bradford *et al.*, 1992). Altogether, it is possible that the sea bass also have developed

alternative regulatory pathways to respond to stress without directly affecting the H-P axis, which could explain why despite dexamethasone administration the animals were still responsive to the stressor.

Even though dexamethasone administration did not prevent the rise in cortisol levels in response to stress, no alterations were seen in testosterone levels.

Considering all the stress experiments that were performed, no correlation was found between cortisol and testosterone. Also, the levels of 11-ketotestosterone measured both in immature and mature fish in all experiments were either below or close to the detection limit of the assay. Similar results have also been obtained in other species, e.g. the degu (*Octodon degus*), a caviomorph rodent, in which the androgen levels (testosterone and 11-ketotestosterone) in males remained low all year, and only at mating, the levels could be detected (Kenagy *et al.*, 1999). Since in all stressful conditions, the 11-ketotestosterone remained mostly undetected, it suggests that there is probably no correlation between cortisol and this steroid either. It is possible, however, that testosterone and 11-ketotestosterone may have been metabolised to other steroids not detected by the assays, which could influence the normal gonadal development.

Nonetheless, whether or not stress can influence the sex differentiation in fish is a question that still remains to be answered. In fact, several possible mechanisms through which cortisol can influence this process exist, including a direct effect of cortisol at one or more sites in the endocrine cascade, or an effect from some other component of the secondary response to stress.

Earlier studies have suggested that the 11-oxyandrogens are potent masculinizing androgens and have also been proposed to be important in sustaining the differentiation and early development of the testes in fish (Guigen *et al.*, 1999). In most fish species, these steroids appear to be testis specific (Guigen *et al.*, 1995). However, it has been shown that some fish larvae, e.g. Arctic charr, are able to synthesize these steroids prior to gonad differentiation (Khan *et al.*, 1994). In rainbow trout fry, treated with equimolar quantities of cortisol and cortisone, the sex ratio was skewed in favor of males, which was suggested to be due to the conversion of these exogenous corticosteroids into 11-oxyandrogens. Since the interrenal tissue is the most steroidogenic active tissue in fish prior to testis development, it has been suggested that the interrenal is the most probable source of precursors for the synthesis of these androgens.

Two types of 11-β HSD isozymes, type 1 and type 2, which are able to catalyse the conversion of 11-hydroxysteroids (e.g. cortisol) into 11-ketosteroids (Quinkler *et al.*, 2001). It has been suggested that cortisol from the adrenals, can be converted into 11β- hydroxyandrostenedione and 11β- ketoandrostenedione and subsequently into 11β- hydroxytestosterone and 11β- ketotestosterone (Goswami *et al.*, 1985), functioning as a source of masculinizing androgens prior to testis differentiation. This hypothesis has gained further support throughout the years since it has been shown that in castrated African catfish (*Clarias gariepinus*), there are detectable levels of 11β- ketotestosterone and 11β- hydroxytestosterone in the plasma (Vermeulen *et al.*, 1995), which further emphasizes that there is a peripheral conversion of, most probably, an adrenal precursor steroid into these two steroids (Vermeulen *et al.*, 1995).

Sex differentiation, in many lower vertebrates, is dependent upon the balance between androgens and estrogens at critical stages during development (Gonzalez and Piferrer, 1999). Steroids given at the appropriate time have been shown to influence sex differentiation both in gonochoristic and hermaphrodite species (Chang *et al.*, 1995). Early exposure of embryos or larvae to estrogens or androgens has proven effective in manipulating the phenotypic gender of the gonads (Patiño *et al.*, 1996; van den Hurk and Cordt, 1985; Kime, 1978; Gilling *et al.,* 1996; Blázquez *et al.,* 1999; Chatain *et al.*, 1999). Such studies indicate that steroid hormone production is essential for the normal expression of gender, which lead to the hypothesis that gonadal sex is determined by the local androgen to estrogen ratios with relatively higher or lower ratios yielding testicular or ovarian development, respectively (Hines *et al.*, 1999).

It has been reported that in some species, e.g. black porgy (*Acanthopargus Schlegeli*), in *Sparidentex hasta* (Kime *et al.*, 1991), and in the anemonefish (*Amphiprion melanopus*) (Godwin and Thomas, 1993), the development of testicular tissue and the levels of 11-ketotestosterone in blood plasma are correlated (Guigen *et al.*, 1995). On the other hand, in several lower vertebrates, e.g. reptiles (Pieau *et al.,* 1999; Desvages and Pieau, 1992; Chardard *et al.*, 1995; Rhen and Lang, 1994; Smith and Joss, 1994), birds (Villalpando *et al.*, 2000) and fish (Chang *et al.*, 1999, Cotta *et al.*, 1999; Kwon *et al.*, 1999, Nagahama, 1999), it has been shown that endogenous estrogens act as natural inducers of ovarian development.

In fact, in most fish species studied, it has been shown that the enzyme responsible for the final conversion of testosterone to estradiol, *CYP* 19 or P_{450}

aromatase, is mainly expressed in the ovarian tissue. In Japanese flounder (*Paralichthys olivaceus*) (Kitano *et al*., 1999), in different tilapia species (Nagahama, 1999; Kwon *et al*., 2001), in rainbow trout (Guigen *et al*., 1998), and in medaka (Scholz *et al*., 2000), *CYP* 19 is highly expressed in the ovary and no expression or only weak expression can be detected in the testis. Similarly, in sea bass the expression of *CYP* 19 mRNA was very high in ovarian tissue and only weakly expressed in testicular tissue.

Nuclear receptors- estrogen receptors (ER) mediate many of the effects of estrogens (Kishida and Callard, 2001; Andrews *et al*., 1997). So far, three distinct ER subtypes (termed ERα, ERβ and ERγ) have been reported in fish (Socorro *et al*., 2000; Pakdel *et al*., 2000; Xia *et al*., 1999; Xia *et al*., 2000; Patiño *et al*., 2000; Hawkins *et al*., 2000; Chang *et al*., 1999). However, there is still no general consensus on the existence of the ERγ, and in addition it has been shown that in fish there are two different ERβ subtypes- ERβ1 and ERβ2. In this study three distinct estrogen receptors were isolated in sea bass, which presented higher similarity to the ERα, ERβ1, and ERβ2 already reported in other fish species.

The estrogen receptors are widely expressed in several tissues, and they have all (ERα, ERβ1, and ERβ2) been detected in the gonads of several fish species. In tilapia and catfish, ERα mRNA is detected both in the testis and ovaries (Chang *et al*., 1999; Xia *et al*., 2000). In sea bream (*Sparus aurata*), ER α mRNA is present in the mature testis but it has not been detected in mature ovaries (Socorro *et al*., 2000; Socorro, 2001). In rainbow trout the ERα mRNA expression has been detected in the ovaries (Nagler *et al*., 2000). In sea bass however, the expression of ERα mRNA was never detected in either gonads, however its presence cannot be excluded, since the levels of this receptor could be below the detection level of RT-PCR and/or restricted to only a few cells.

In sea bream (*Sparus aurata*) (Socorro *et al*., 2000; Socorro, 2001), in eel (Tchoudakova *et al*., 1999), in the channel catfish (Xia *et al*., 2000), and in tilapia (Chang *et al*., 1999), the ERβ1 was clearly expressed in ovary and testis. Similarly, in sea bass, the ERβ1 mRNA was expressed in both types of gonads. Nonetheless, the expression of this receptor was a lot higher in ovaries than in testis, in which only a faint signal could be detected. These results are consistent with the expression of *CYP* 19 mRNA in mature gonads, which suggest that this receptor most probably has a determinant role on the regulation of the enzymatic activity of aromatase, and thereby

could probably be implicated in maintaining the differentiation and the specific steroidogenic activity in this tissue.

On the contrary, in sea bass, the expression of the ERβ2 mRNA was a lot higher in the testis than in the ovaries, which points out that probably, these two receptors have different physiological roles in fish.

In most fish species, *CYP* 11β1 or P_{450} 11β-Hydroxylase, one of the key enzymes involved in the conversion of testosterone to 11-ketotestosterone, is mainly expressed in testicular tissue. In rainbow trout (*Onconrhynchus mykiss*) (Liu *et al.*, 2000; Baroiller *et al.*, 1999), and in eel (*Anguilla japonica*) (Jiang *et al.*, 1998), *CYP* 11B1 mRNA is highly expressed in testicular tissue, and no expression or only weak signals can be detected in ovarian tissue. In sea bass, although *CYP* 11B1 mRNA expression was apparently higher in testicular tissue than in ovaries, the difference was small, suggesting that this enzyme may not be as determinant as aromatase.

The action of androgens is mediated by the androgen receptors. So far, two different isoforms of AR (ARα and ARβ) have been identified in fish (Touhata *et al.*, 1999; Takeo and Yamashita, 1999; Sperry and Thomas, 1999; Morrey and Nagahama, 1999; Sperry and Thomas, 1999; Ikeuchi *et al.*, 1999; and Todo *et al.*, 1999). In this study, however, only one receptor was isolated that possessed higher similarity to the subtype β (ARβ).

The ARβ mRNA has been shown to be expressed in both testis and ovaries in some fish species, e.g. in eel (Ikeuchi *et al.*, 1999) and *Thalassoma duperrey* (Morrey and Nagahama, 1999). Similarly, in sea bass, ARβ mRNA was expressed in both gonads, whereas the expression of this receptor in the testis was very high, and in ovary it was only weakly expressed. These results are in accordance to recent reports in *Thalassoma duperrey*, in which not only this receptor was more expressed in the testis, as also its expression was shown to be correlated to the development and proliferation of the testicular tissue (Morrey and Nagahama, 1999). Moreover, the higher expression of ARβ mRNA in the testis is consistent with the higher expression of *CYP* 11B1 mRNA also in the testis, which, further emphasizes the previous suggestion that in sea bass the enzyme 11β-hydroxylase is probably involved in maintaining testicular differentiation and steroidogenic activity, most probably through interaction/activation of the androgen receptor.

Assuming that gonadal sex is determined by the local androgen to estrogen ratios, then a more or less clear sexual dimorphism of plasma profiles of sex steroids and/or enzymatic expression around the period of sexual differentiation would be expected. For this reason the expression of all isolated genes was determined in sea bass during the steroid sensitive period.

Before the steroid sensitive period starts (80 dph), there is a high expression of both *CYP* 11B1 and ARβ mRNAs, after which the expression decreases. *CYP* 11B1 mRNA expression showed higher expression and higher dispersion of values before the androgen sensitive period started, and throughout this period the expression is similar in all individuals and no dimorphic profile can be observed. On the contrary, the expression of ARβ mRNA presents a highly dimorphic pattern before and during the androgen sensitive period, and the expression of this receptor is only similar in all individuals, when this period ends.

It has been shown that in this species, the critical period for androgen-inducible masculinisation is between days 96 and 126 post-fertilization (Bláquez *et al.*, 2001). Thus, several studies where the administration of androgens during this period of development was performed, all-male populations were obtained (Chatain *et al.*, 1999; Blázquez *et a*l., 2001; Blázquez *et al.*, 1995). In fact, in this study the expression of CYP 11B1 and ARβ mRNAs are higher during this period, which suggests that indeed after 130 dph the larvae are most likely less sensitive to androgen treatments. In addition, the dimorphic expression profile of ARβ mRNA, together with the higher expression shown in mature testicular tissue than in ovaries suggests that the fish, which presented higher expression, will have a higher probability of differentiating as males.

Recently, several reports have described the expression of *CYP* 19 mRNA during sexual differentiation in several species. In chicken, *CYP* 19 mRNA is detected in the gonads of genetic females but not in males during sexual differentiation (Yoshida *et al.*, 1996; Nakabayashi *et al.*, 1998); in reptiles an amphibians aromatase activity is significantly higher during ovarian differentiation (Desvages and Pieau, 1992; Chardard *et al.*, 1995); in tilapia (*Oreochromis niloticus*) positive immunostaining against aromatase appears in differentiating ovaries, but not in differentiating testis (Chang *et al.*, 1997).

In addition, it has been shown that in tilapia (*Oreochromis niloticus*) (Kwon *et al.*, 2001), and in the Japanese flounder (*Paralichthys olivaceus*) (Kitano *et al.*, 1999),

there is a sexual dimorphism of *CYP* 19 mRNA expression, in that in females, the expression level is maintained or increased throughout ontogeny, while the level in males is always low (Kwon *et al.*, 2001). In sea bass, during the period of androgen sensitivity, *CYP* 19 mRNA was very low expressed in all individuals, and after day 130 dph the expression increased. However, no dimorphic pattern in the expression could be observed. Nonetheless, considering that *CYP* 19 mRNA expression only started to increase at the end of period that was analysed in this study, and that this increase was accompanied by the increase of expression of the ERβ2 mRNA, it suggests that if there is a dimorphic expression in sea bass it is more likely to be detected after 170 dph.

The expression of ERα and ERβ1 mRNAs was also studied in sea bass. ERα mRNA was never detected at any stage of development. On the contrary, ERβ1 mRNA was expressed independently of the stage of development, but no differences in level of expression could be detected. Similar results were also obtained in immature sea bream (*Sparus aurata*), in which ER α mRNA could not be detected until the gonads were fully differentiated but ERβ1 mRNA was always expressed throughout development with no differences in expression (Socorro, 2000; Socorro *et al.*, 2001). Thus, the presence of ERβ1 and not ERα mRNAs in immature gonads of sea bass suggest a possible role for ERβ1 gene in gonadal development.

The correlation analysis performed with the expression of the various enzymes and receptors obtained in this study point out that the decrease in the expression of *CYP* 11B1 and ARβ mRNAs after 130 dph is correlated with the increase in the expression of the ERβ2 mRNA. These results suggest that in sea bass, the high sensitivity to androgens between 96 and 126 dph is most likely due to the low expression of ERβ2 mRNA. Similar results have also been obtained in different vertebrate species. In reptiles, e.g. lizards, changes in the levels of sex hormones and their related receptors appear to be correlated with testicular regression, so that when estrogens appear, androgens fall (Andó *et al.*, 1992). In fact, it has been shown that in mature male lizards (*P. sicula*), there is a marked down- regulatory effect of estrogens on AR mRNA expression. In contrast, there was never a positive or negative effect of androgens upon the expression of ER mRNA (Cardone *et al.*. 1998).

In fish, e.g. rainbow trout, expression of *CYP* 19 mRNA is highly expressed in female gonads 2 weeks before the first sign of histological differentiation (Guigen *et al.*, 1998), whereas in male rainbow trout, the expression is detected but a few hundreds

times less than in female gonads (Guigen *et al.*, 1999). Conversely, *CYP* 11B1 mRNA is highly expressed in male gonads (Baroiller *et al.*, 1999). In addition, in this species, treatment with estrogen in genetic all male populations, significantly decreased the levels of expression of *CYP* 11B1 mRNA, which also suggested that the inhibition of the synthesis of testicular androgens may be an important step required for the active feminisation of genetic males (Govoroun *et al.*, 2001). Similar results have also been reported in sex reversing fish, e.g. in sea bream (Condeça and Canario, 2001) and in black porgy (Chang *et al.*, 1997), in which estrogen treatment caused a marked reduction of 11β-Hydroxylase activity, and thus a strong inhibition of testicular growth These results led the authors to suggest that the effect of estrogen in these species is to increase the proportion of ovarian tissue at the expense of testicular (Condeça and Canario, 1999), most probably due to the shift of 11-ketotestosterone synthesis through aromatase activity (Chang *et al.*, 1997).

Sex steroids have local, direct effects on germ-cell development, but also act as endocrine hormones to influence other cell types and organs involved in sex differentiation. This multilevel control is very complex in order to provide the necessary plasticity for gonadal development to proceed in context with intrinsic and external factors (Devlin and Nagahama, 2002). This complexity also provides many levels at which sex differentiation can be disrupted. In this work, it was assumed that administration of dexamethasone during the period that has been established as being the time during sexual differentiation at which fish present highest sensitivity to androgen treatments, would most likely be the period at which the hypothesized adrenal androgens would influence sex differentiation in this species. However, considering that the expression of *CYP* 11B1 and ARβ mRNAs were higher before this period (80 dph), and that in this work dexamethasone was administered latter on (100 dph), it is most likely that the observed alterations to the normal development of the gonads in treated fish is an indication that this drug should have been most probably administered earlier. Moreover, since the expression of *CYP* 11B1 and ARβ mRNAs were shown to be correlated to the expression of ERβ2 mRNA, it is probable that if dexamethasone had been administered earlier in development, the expression of *CYP* 11B1 mRNA would have been probably reduced or inhibited at the beginning of the period of sexual differentiation, and the expression of *CYP* 19 and ERβ2 mRNAs would probably increase earlier during sexual development and thereby the sex ratios might have been

skewed towards the female direction. Nonetheless, further work is still necessary to establish a relation between the interrenal tissue and sexual differentiation in sea bass.

V- FINAL CONSIDERATIONS

The underlying hypothesis of this thesis was that in intensive culture, the sea bass interrenal tissue overproduces glucocorticoids in response to stress, which is accompained by the production of adrenal androgens, and thus shifting the normal androgen/ estrogen production and consequently skewing the sex ratios in favour of males.

Dexamethasone was shown to be effective in suppressing the HPI axis in juvenile sea bass. Administration of 75 mg Kg^{-1} food dexamethasone to fish during the period of sexual differentiation did not alter the sex ratios in sea bass (60-65% males; 35-40% females), however, 150 mg Kg^{-1} food dexamethasone induced some alterations to the normal development of the gonads. In this respect, 7% of the fish had testicular tissue with scattered intra testicular oocytes and 4% of the fish were still undifferentiated at 10 months of age.

Nevertheless, the use of this synthetic glucocorticoid induced significant alterations to the relationship between length and weight (condition factor) in a dose dependent manner. Thus, the higher the dose of dexamethasone, the lower the condition factor was obtained. These results point out that the doses of dexamethasone had significant side- effects, and by thus altering the normal growth of the fish.

Despite the referred effects of dexamethasone on gonad differentiation, still no conclusive data was obtained in order to establish a relationship between the interrenal and sexual differentiation. In fact, all stress experiments performed failed to establish a correlation between the elevated levels of cortisol and androgen levels (testosterone and 11-ketotestosterone). Altogether it is suggested that even though cortisol may be influencing the sexual differentiation, it most probably not through its direct influence on testosterone or 11-ketotestosterone levels.

Three steroidogenic enzymes (*CYP* 1 and *CYP* 11B1) and four steroid receptors (ERα, ERβ1, ERβ2 and ARβ) were isolated in sea bass.

In the ovarian tissue, the expression of *CYP* 19, ERβ1 was very high and in the testicular tissue they were only weakly expressed. These results suggest that this receptor most probably has a determinant role on the regulation of the *CYP* 19 activities, and thereby could probably be implicated in maintaining the differentiation and the specific steroidogenic activity in this tissue. On the contrary, the expression of *CYP*

11B1, ERβ2 and ARβ was higher in the testicular tissue than in the ovarian tissue. The different expression of the ERβ1 and ERβ2 in the testis and in the ovaries, suggest that these two receptors most likely have different physiological roles in fish. Moreover, the higher expression of ARβ and *CYP* 11B1 in the testis suggests that in sea bass, *CYP* 11B1 is probably involved in maintaining testicular differentiation and steroidogenic activity, most probably through interaction/activation of the ARβ. The expression of ERα was never detected in either gonad, which suggests that this receptor is probably not essential for the activity and/or maintenance of the differentiated state of the gonadal tissue.

Assuming that gonadal sex is determined by the local androgen to estrogen ratios, then a more or less clear sexual dimorphism of the expression of *CYP* 19, *CYP*11B1, ERα, ERβ1, ERβ2 or ARβ around the period of sexual differentiation would be expected.

Before the steroid sensitive period starts (80 dph), the expression of both *CYP* 11B1 and ARβ was high, but decreased throughout this period. On the contrary, the expression of *CYP* 19 and ERβ2 was always low throughout the androgen sensitive period, but towards the end of this period the expression progressively increased. The expression of ERβ1 was similar in all individuals throughout the androgen sensitive period. The expression of ERα was never detected throughout the androgen sensitive period, which suggests that it is probably not essential for gonadal differentiation at this stage of development.

Comparing the expression profiles of all the genes studied, a negative correlation between the expression of *CYP* 11B1 and ARβ and the expression of ERβ2 was found. Nonetheless, only the ARβ presented a dimorphic pattern of expression throughout the androgen sensitive period, which suggests that this gene most likely has a fundamental role in sexual differentiation in sea bass.

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