Universidade do Algarve

Unidade de Ciências e Tecnologias

dos Recursos Aquáticos

CONTROLE HORMONAL DA DIFERENCIAÇÃO E INVERSÃO SEXUAL NA DOURADA *Sparus aurata* (L.)

Hormonal control of sex differentiation and reversal in sea bream *Sparus aurata* (L.)

Dissertação apresentada à
Universidade do Algarve para
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AGRADECIMENTOS

Uma tese a fogo lento
obrigou a muito tino
se não a acabo ainda rebento
muito obrigado Adelino

Muita hormona tive em mente
e muito houve que eu não li
muitos RIAs é ponto assente
muito obrigado Debbie

No laboratório à procura
muita se busca mas pouco muda
doutorando tem vida dura
pede-se à Elsa que ela ajuda

No café com o Vília e o Guerreiro
não há tréguas e não se diz bem
assim nos ensinou Mario o "matreiro"
o Faustino e a Rute já aprenderam também

Para a Sílvia e Cecília PCR é maçada
e a Modesto só fala do xarroco
o Cavaco protege a sua bancada
o Pepe e o Juan acham que ele é louco

Outros há que também habitam
no laboratório dois vinte e oito
para todos eles a minha gratidão
e também para o João Reis sempre tão afoito

E aos amigos que tanto perguntam
então João já acabaste?
há coisas que nunca mudam
espero que esta tese lhes baste

Para a frente é que é caminho
assim dizia a Teresa Palaré
a ela agradeço com carinho
de ter remado contra a maré

Johnny Kondeka

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LIST OF ABBREVIATIONS*

DA	dopamine
dpf	days- post- fertilization
dph	days- post- hatch
GABA	gamma-aminobutiric acid
GC-MS	gas chromatography- mass spectometry
GnRH	gonadotropin-releasing hormone
GSI	gonadosomatic index
GtH	gonadotropin
hr	hour(s)
H&E	hematoxylin and eosin
hCG	human chorionic gonadotropin
HUFA	highly unsaturated fatty acid
...-Hyd	...- hydroxylase
...-HSD	...-hydroxysteroid dehydrogenase
I. U.	international unit
mRNA	messenger RNA
NAD	β-nicotinamide adenine dinucleotide (reduced form)
NPY	neuropeptide Y
OTMS	oxime-trimethylsilyl
PGC	primary germ cell
RIA	radioimmunoassay
5β-Red	5beta-reductase
RPTLC	reverse phase thin layer chromatography
sbER(α/β)	sea bream estrogen receptor (alpha/beta)
SEM	standard error of the mean
RPTLC	reverse phase thin layer chromatography
TLC	normal phase thin layer chromatography
TMS	Trimethylsilyl
3α.11ßA5ß-17one	3α.11ß-dihvdroxv-5ß-androstan-17-one
3α,17αA5β-11one	3α,17β-dihydroxy-5β-androstan-11-one
3β,11βA5α-17one	3β,11β-dihydroxy-5α-androstan-17-one
3α,17,20αP5β-11one	3α,17α,20α-trihydroxy-5β-pregnan-11one
3α,11β,17,20αP5β	3α,11β,17α,20α-tetrahydroxy-5β-pregnane
3α,17,20β,21P5β	3α,17α,20β,21-tetrahydroxy-5β-pregnane
3α,11β,17,21P5β	3α,11β,17α,21-tetrahydroxy-5β-pregnane
11β,17αP-3,20dione	11β,17α-dihydroxy-4-pregnene-3,20-dione
ethE$_2$	17α-ethynilestradiol
estriol	1,3,5(10)-estratriene-3,16α,17β-triol

* for steroids see also Table 3.1

SUMÁRIO

A dourada, *Sparus aurata*, é um peixe hermafrodita protândrico, i.e, atinge a primeira maturação sexual como macho podendo inverter o seu sexo para fêmea nas épocas de reprodução subsequentes. O fenómeno, designado como inversão sexual, sendo bastante comum nos teleósteos em geral, é ainda mal conhecido, sobretudo do ponto de vista genético e fisiológico (desconhecem-se, nomeadamente, quais e como se relacionam as moléculas indutoras e mediadoras do processo). É bastante difícil compreender que os mesmos factores genéticos possam regular ciclicamente o aparecimento dos dois sexos na gónada e conceber um modelo onde os mesmos factores possam interactuar com factores sociais ou ambientais que se sabem poder ter influência na expressão do sexo. Deste modo o hermafroditismo tem sido mais considerado como um processo de (re)diferenciação sexual do que de determinação sexual como, por exemplo, nos mamíferos. De um modo geral, o cérebro é considerado como o centro de controle de todos os fenómenos ligados à reprodução e capaz de integrar toda uma série de estímulos, quer de natureza interna (p. ex. acção retroactiva de esteróides sexuais) quer externa (p. ex. fotoperíodo, temperatura, feromonas etc). A resposta surge depois na forma de diversos tipos de moléculas estimuladoras ou inibidoras da produção de gonadotrofinas por parte da pituitária que, por sua vez, controlam a esteroidogénese e, consequentemente, a gametogénese. No entanto, nos peixes, o perfil de esteróides sexuais varia consideravelmente de espécie para espécie o que dificulta o conhecimento do papel fisiológico da maioria desses esteróides na diferenciação/inversão sexual. Deste modo o objectivo geral desta tese é procurar relacionar o perfil de esteróides sexuais da dourada com o processo de inversão e /ou diferenciação sexual para gerar informação que contribua para um modelo geral para a compreensão do hermafroditismo na reprodução dos peixes.

O processo de inversão sexual foi seguido em peixes feminizados artificialmente por administração de estrógenio na ração e também em peixes a completar 2 anos de idade e potencialmente em inversão sexual natural. Em ambas as experiências se verificou que a feminização da gónada (completa no primeiro caso e parcial no segundo) se processou à custa da inibição de desenvolvimento da parte testicular sem que se tenha verificado um aumento real do ovário. Na inversão natural foi mesmo

possível detectar a existência de focos degenerativos do testículo onde as espermatogónias foram substituídas por massas de tecido conjuntivo de aparência amarelo-castanho não relatadas anteriormente nesta espécie. O nível de estrógenio no plasma sanguíneo foi sempre muito baixo embora na inversão sexual induzida a capacidade de produzir estradiol-17β (E_2), tal como a testosterona, a partir da androstenediona (medida por radioimunoensaio) se verificasse inversamente proporcional à proporção de tecido testicular. A capacidade esteroidogénica das gónadas feminizadas (utilizando 17α-hidroxiprogesterona radioactiva como precursor) revelou um paralelismo significativo com as mudanças morfológicas. Ou seja, a atrofia do testículo foi sobretudo mais acompanhada pela diminuição de alguns fluxos metabólicos, nomeadamente na produção de 11β-hidroxiandrostenediona (11βA) e etiocolanolona (3αA5β), do que pelo aparecimento de novas vias enzimáticas. A presença de elevados níveis de 11βA foi confirmada *in vivo* assim como a de esteróides 5β-reduzidos (incluindo um de propriedades cromatográficas semelhantes a 3αA5β) o que sugere um possível papel para estes esteróides na espermatogénese desta espécie. Foi detectada a actividade de 6(α/β)-hidroxilase que são enzimas esteroidogénicas muito pouco comuns em peixes, embora a sua acção não estivesse relacionada de um modo evidente com a inversão sexual.

Ao nível ontogénico verificou-se que a gonadogénese ocorre por volta dos três meses de idade (87 dias pos-eclosão) e não é afectada pela exposição ao estrógénio exógeno. A gametogénese verificou-se ter início cerca de 1 mês mais tarde do que em anteriores descrições, por volta dos 5 meses, através da formação das ovogónias junto à cavidade central e como consequência da formação de ninhos de células germinais primordiais. A exposição faseada das larvas ao estrogénio demonstrou a sua insensibilidade a esta hormona durante os primeiros 100 dias de vida apesar da técnica utilizada de incorporação de E_2 nas presas vivas (rotíferos e artémias) ter sido satisfatória. A causa para este facto pode dever-se à metabolização do E_2 noutros esteróides biologicamente inactivos ou mais provavelmente à inexistência da forma α do receptor de estrogénio da dourada, que se pensa estar envolvido na inversão sexual, nas larvas destas idades.

De um modo geral, esta tese demonstra que o E_2, apesar de revelar baixos níveis endógenos no plasma sanguíneo durante as primeiras fases de inversão sexual (antes da iniciação da vitelogénese) é uma hormona largamente associada a inversão sexual na dourada pois é susceptível de induzir a mesma e também porque se verifica maior capacidade para a sua síntese nas gónadas em feminização. No entanto, o efeito da administração de estrógénio parece ser dependente do estado de desenvolvimento da gónada porquanto a gónadas muito indiferenciadas não invertem o seu sexo. O conjunto de resultados dá também ênfase à ideia de que a teoria da primazia de diferenciação sexual feminina geralmente aceite nos mamíferos possa também ser aplicada aos peixes apesar da existência de casos de hermafroditismo protândrico. Apesar das douradas se diferenciarem como machos para a primeira estação de reprodução existe previamente a formação de um ovário efémero que depois degenera. Para alem desta evidência morfológica, também do ponto de vista fisiológico, ao nível da capacidade esteroidogénica das gónadas, parece haver uma via metabólica feminina de base que só é ultrapassada se algum mecanismo masculinizante ocorrer e que retorna a condição feminina quando esse mecanismo activo cessar.

CHAPTER I

GENERAL INTRODUCTION

I General Introduction

I.1 Characteristics of the species

The gilt-head sea bream, *Sparus aurata*, is a marine teleost with a silvery grey ovoid body and a large dark patch localised at the anterior end of the lateral line. One of most distinctive features of this species is a golden curved bar across the forehead, bordered by two dark zones, which gives the species its common name (Fig. 1.1). Body length rarely exceeds 70cm but it usually reaches 30-35cm length. Generally, this fish can be found, solitary or in small groups, in littoral waters with a sandy bottom and lagoons. It is mainly carnivorous (molluscs, crustaceans and fishes), although sometimes feeds on plants. This species distribution spreads throughout the Mediterranean, where it is rather common, and the Atlantic, from Great Britain to Cape Verde and the Canary islands (UNESCO, 1986).

I.2 Taxonomic Classification

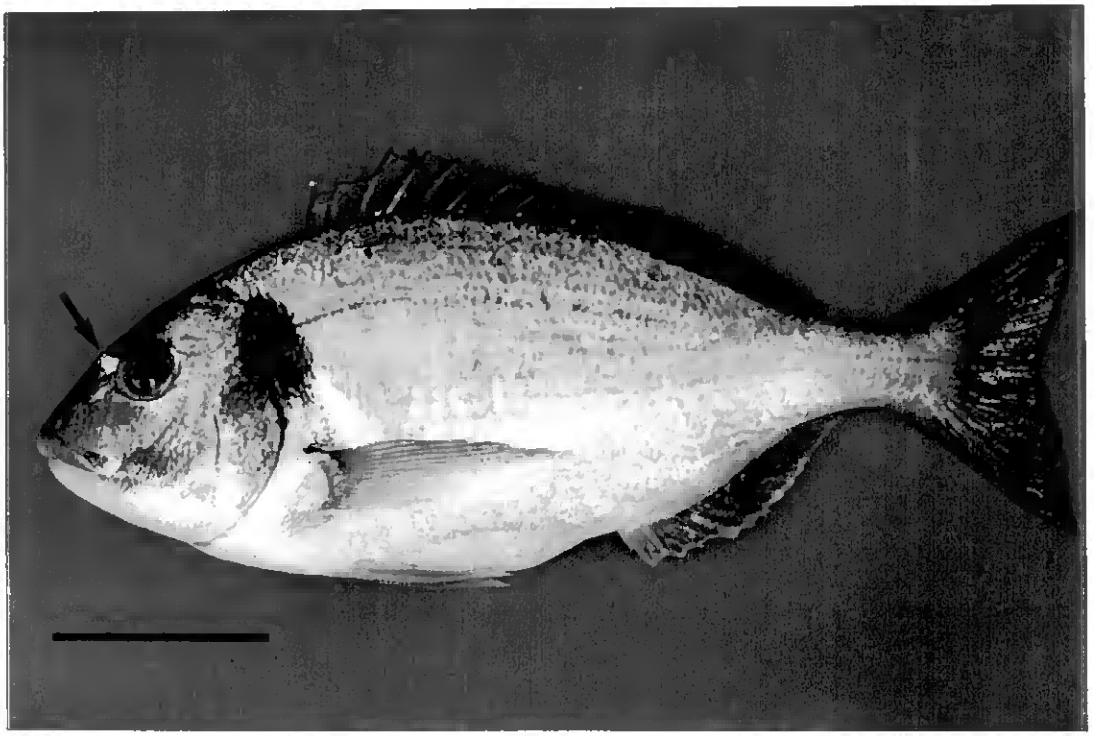
The gilt-head sea bream taxonomic classification is as follows (Nelson, 1994):

Superclass- *Osteichtyes*
Class- *Actinopterygii*
Subclass- *Teleostei*
Superorder- *Acanthopterygii*
Order- *Perciformes*
Suborder- *Percoidei*
Superfamily- *Percoidea*
Family- *Sparidae*
Genus- *Sparus*
Species- *Sparus aurata* (Linnaeus, 1758)

I.3 Economical and Scientific Importance

Sea bream aquaculture techniques were developed during the late seventies and early eighties and, nowadays, it is possible to control all stages of the sea bream life cycle, which is fundamental for profitable fish farm production. This production has been continuously increasing over the last ten years. Total European catches raised from 12280 tons in 1994 to 33226 tons in 1997 (FEAP, 1997) and the main origin of production are Mediterranean countries such as Greece, Italy, Spain and Portugal. Therefore, sea bream is not only becoming economically important but is also readily available, providing two good practical reasons to study this species.

Moreover, in contrast to other vertebrates, teleost fishes exhibit a wide variety of different types of ambisexuality, including protogynic and protandric hermaphroditism (as is the case of the sea bream), making them a group of great interest for investigating sex development in vertebrates (Reinboth, 1982). The genetic basis of hermaphrodites as the sea bream is unknown. In fact, hermaphroditism has generally been considered to be largely a process of sex differentiation (and/or inversion) rather than sex determination as the involvement of possible genetic factors in this process is poorly understood (Price, 1984). It is difficult to understand how the same genetic factors can regulate cyclically the appearance of both sexes in one fish and also to conceive a model where genetic factors can interact with other factors, as sexual steroids, known to be related with sexual differentiation (Borg, 1994). The conflicting data on the effects of steroids in ambisexual fish add to the uncertainty as to whether or not sex hormones have a causative influence on the process of sex inversion (and differentiation) (Reinboth, 1988). Further studies on the sex steroid profile of ambisexual species are needed to improve the current knowledge on the physiological role of sex steroids on sex differentiation and reversal.



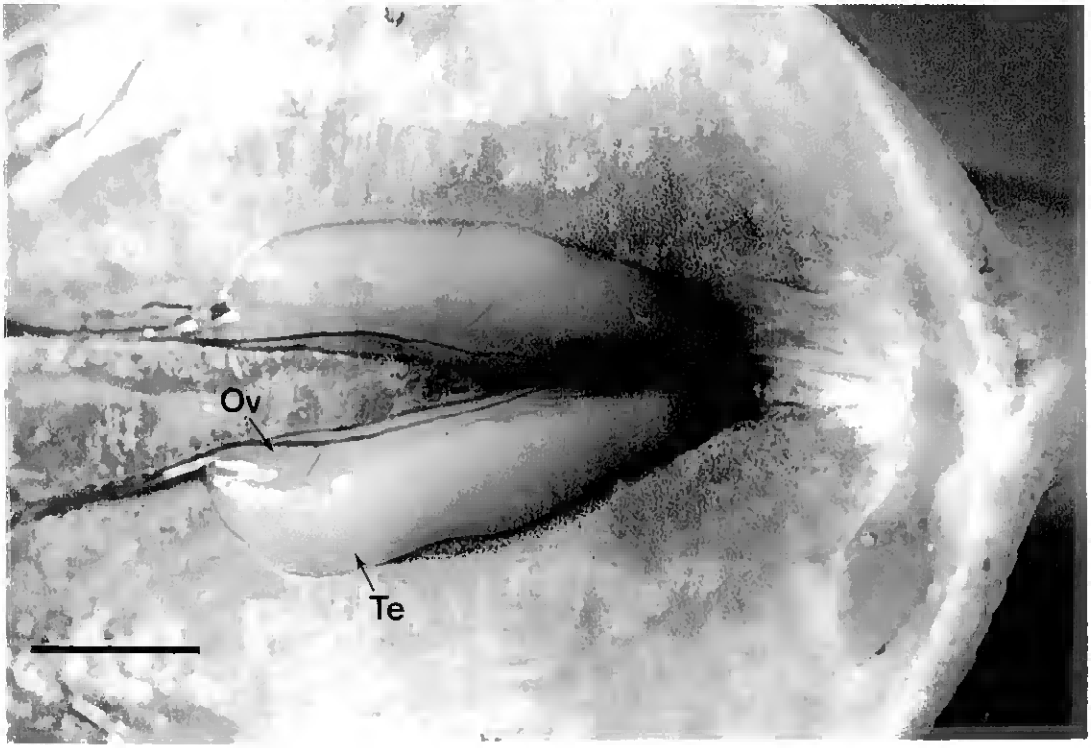


Figure 1.1 (upper photo) – Lateral view of 300g adult sea bream, *Sparus aurata*, exhibiting the most distinctive feature of this species: the golden curved bar across forehead (arrow). Bar corresponds to 5cm.

Figure 1.2 (lower photo)– Sea bream ambisexual gonad, united caudally, located below the dorsal wall of the peritoneal cavity. Note the simultaneous presence of testes (Te) and ovary (Ov). Bar corresponds to 1cm.

I.4 Reproductive biology

The sea bream is a protandrous hermaphrodite, i.e., individuals spawn as males during the first breeding season but may undergo a sex change to female in one of the subsequent spawning seasons (D'Ancona, 1941; Pasquali, 1941). Although there is either testicular (first) or ovarian (later) predominance in sea bream gonad at spawning, both germinal tissues are present during the whole gonadal cycle. Sea bream gonads are paired elongated organs placed along the dorsal wall of the peritoneal cavity just bellow the swim bladder. The gonads unite caudally to form a common genital duct that exits the body between the anus and the urinary pore (Fig. 1.2). According to Reinboth (1982) the fact that the teleost genital apparatus is not connected in either sex with the excretory system, permits, from a morphological point of view, the protandric and protogynic sex reversals observed in these fish. The spawning season in southern Portugal is from October/November to January/February although it can be advanced or delayed several months by temperature and photoperiod manipulations in artificial environments.

I.4.1 Sex differentiation

Gonadogenesis is thought to occur around 90 days post hatch (Power, D.M. unpublished data; see chapter V) although gametogenesis takes place much later. Zohar *et al* (1978) followed the development of the sea bream gonad during the first two years of life. The first germ cells to differentiate are the oogonia, which arise from primordial germ cell (PGC) nests that, unevenly, border the vicinity of the central cavity (see chapter V) at the age of 4 months. One month later, a topographic differentiation becomes evident and the gonad divides into a dorsal region containing the central cavity which will form the ovary and a ventral region, separated by connective tissue, forming the future testis. Until the age of 8 months the oogonia proliferate forming an ephemeral ovary that starts to degenerate simultaneously with a process of active spermatogenesis, leading the gonad to function as a testis in the first (Zohar *et al.*, 1978) or second breeding season (Bruslé-Sicard and Fourcault, 1997). The sea bream testis is of a lobular type, i.e, germ cells are scattered along lobules and the cysts, which remain roughly in the same place during

spermatogenesis, and release the spermatozoa into a central lumen (*vas eferens*) that comunicates with the sperm duct (*vas deferens*) (Billard *et al.*, 1982). The subsequent period is characterized by the start of sex reversal that affects up to 80% of the males (Zohar *et al.*, 1978) but that can be delayed for later seasons (Bruslé-Sicard and Fourcault, 1997). As sex inversion proceeds the lobular organization is disrupted and degenerative processes including phagocytic activity result in testicular regression. In contrast, the ovary recovers its oogenic capacity and develops the ovigerous lamellae increasing the ovarian component of the gonad. However, caution must be taken when diagnosing histologically sex reversal, because protandry involves coexistence of both sexes during the whole life cycle (Yeung and Chan, 1987). Also, sea bream gonads are very plastic and unexpected abrupt sex changes have been reported (Kadmon *et al.*, 1985), emphasizing the need of more reliable histological criteria for sex reversal recognition (Bruslé-Sicard and Fourcault, 1997). Sea bream is a cyclical breeder and therefore its ovary, like most teleosts, contains oocytes at all stages of development, i.e, it is an asynchronous ovary (Nagahama, 1983). Simultaneously with the progressive dominance of the ovary in the gonad, oocytes enter a growth phase, vitellogenesis, before the resumption of meiosis. Oocyte maturation is the following event and consists of germinal vesicle breakdown, chromosome condensation and assembly of the first polar body, all of which are prerequisites for ovulation and successful fertilization (Nagahama, 1997).

I.5 The role of steroids in the Brain-Pituitary-Gonad axis

I.5.1 Teleosts in general

It is now very well established that sexual steroids acting on the gonads are not the only factors regulating fish reproduction and both the brain and the pituitary are involved in this process (Dufour *et al.*, 1999; Kah *et al.*, 1999). The brain is one of the main organs controlling reproduction and is capable of perceiving both internal (e.g feedback of steroid hormones) (Francis *et al.*, 1993; Soga *et al.*, 1998) and external stimuli such as photoperiod (Amano *et al.*, 1995; Senthilkumaran and Joy, 1995), temperature (Shimizu, 1996), social

signals (Francis *et al.*, 1993; Rissman, 1996) or pheromones (Christensen and Sorensen, 1996). For example, in sea bream a significant inhibition of sex inversion was observed when a group of young males was mixed with larger females (Happe and Zohar, 1988). The brain response to these stimuli can be made by several stimulatory or inhibitory molecules of gonadotropin synthesis but the most important seem to be gonadotropin–releasing hormones (GnRH, stimulatory), dopamine (DA, inhibitory) and γ-aminobutyric acid (GABA, modulatory) (Trudeau and Peter, 1995; Kah *et al.*, 1999). These substances control gonadotropin release from the pituitary, GtH I and GtH II (homologous to the follicle stimulating hormone, FSH, and the luteinizing hormone, LH, respectively, in tetrapods) which, in turn, control gametogenesis and steroidogenesis (Fig. 1.3). In teleosts, however, differences in the pubertal profiles of GtH I and GtH II suggests species-specific variations in the regulation of gonadotropin synthesis and their respective roles in the initiation of puberty, gametogenesis and steroidogenesis (Dufour *et al.*, 1999). As a consequence of the variety of reproductive strategies in teleost fish, the role of steroid hormones as major mediators of sexual differentiation and gametogenesis are complex and also vary considerably. Nevertheless, knowledge on the mechanism of action of some critical steroids with known functions in several teleosts, e.g. estradiol-17β (E_2; ovarian development and oocyte growth), 11-ketotestosterone (11KT; spermatogenesis) and 17α,20β-dihydroxy-4-pregnen-3-one (17,20βP; gamete maturation), can provide valuable insight into the evolution of the reproductive function (Nagahama, 1999).

The synthesis of gonadal steroids through GtH stimulation is generally mediated by specific cells. In the testis, interstitial cells (homologous to mammalian Leydig cells) occur between testicular lobules and are considered to be the major site of androgen synthesis (Nagahama *et al.*, 1982) but boundary lobular cells, or Sertoli cells, are also of major importance for spermatogenesis. Germ cells are structurally and functionally supported by Sertoli cells, which are also considered to mediate androgen effects on germ cells (Shulz *et al.*, 1999). In the preovulatory ovary, the main steroidogenic role is attributed to the follicle layers, i.e., the thecal and granulosa cells. In common with mammals, in teleosts these cells interact in a "two-cell-type" model to establish a steroid biosynthetic pathway (Kagawa *et al.*, 1982). Furthermore, E_2 and 17,20βP, two major steroids in oocyte development, are

synthesized by the granulosa layer after their precursors have been produced in the theca layer (Nagahama, 1999).

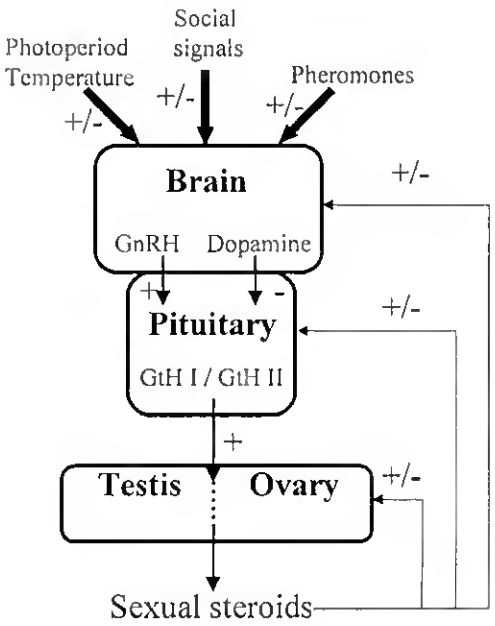


Figure 1.3 – Simple schematic representation of the cascade of reproductive events acting on the brain-pituitary-gonad axis in teleosts. GnRH, gonadotropin-releasing hormone; GtH, gonadotropin; +, stimulatory effect; -, inhibitory effect; +/- possible stimulatory and/or inhibitory effects.

Sex steroids can have complementary physiological roles as a consequence of their action at multiple sites (Kah *et al.*, 1999) or else their physiological role may involve stimulatory or inhibitory effects on other steroids (Antonopoulou *et al.*, 1999). For example, in the proposed endocrine mechanism of sex reversal in protandrous black porgy, *Acanthopagrus shlegeli*, E_2 is thought to act through a positive feedback to stimulate GnRH and GtH synthesis and release, which in turn stimulates aromatase activity to produce more E_2 (Chang *et al.*, 1997). In the Atlantic salmon, *Salmo salar*, silastic implants of testosterone (T) prevented the normal testicular decline after the breeding season by interfering at the level of GtH, which affects the steroidogenic axis and hence the gonad status (Antonopoulou *et al.*, 1999). The mechanism proposed was that exogenous T induced a positive feedback on GtH II which prevented reduction in plasma 17,20βP and, in turn, the suppression of Sertoli cell phagocytic activity upon the spermatozoa, prolonging the spermiation period.

I.5.2 The hermaphrodite Sea Bream

The number and type of GnRHs, may vary among species. In the sea bream, three different forms have been isolated from the brain, e.g. salmon (s)GnRH, chicken (c)GnRH and sea bream (sb)GnRH (Gothilf *et al.*, 1996) but only sbGnRH and cGnRH reached the pituitary (Holland *et al.*, 1998). Furthermore, sbGnRH (and GtH II) pituitary content was 3-17 fold higher in both male and females with developing gonads, but not in recrudescent bisexual gonads, suggesting that sbGnRH is the form which controlls reproduction in this species (Holland *et al.*, 1998). Other substances with GnRH-like activity were, however, detected in the sea bream ovary (Nabissi *et al.*, 1997).

An assay for sea bream GtH I has not been developed yet but the genes for β-GtH I and II were found to be differentially expressed in male and female sea bream (Elizur *et al.*, 1996). Mature males appeared to have higher GtH I mRNA levels than mature females while the opposite is true for GtH II mRNA levels. Pituitary and plasma GtH II levels showed, however, different profiles since in mature females levels of both were high, whereas in mature males only the plasma content was high (Holland *et al.*, 1998). Furthermore, plasma GtH II gradually increased during both the male and female reproductive cycle. The temporal differences in pituitary GtH II content further supported the hypothesis that GtH I and II are regulated differently and their roles in male and female sexual development may, therefore, not be equivalent (Holland *et al.*, 1998).

There is no evidence to show that sex steroids are stimulated directly by sea bream gonadotropins at any of their characteristic sexual stages. Nevertheless, pioneering studies performed by Zohar and his co-workers have shown that low doses of human chorionic gonadotropin (hCG) induced maturation, ovulation and spawning, for at least 4 days, in female sea bream in the last stages of oocyte vitellogenesis (Zohar and Gordin, 1979). Furthermore, hCG treatment *in vitro* enhanced 11KT in both ovary and testis, 11β-hydroxy-testosterone (11βT) and T in the ovary whereas T synthesis was strongly inhibited in the testis (Eckstein *et al.*, 1978). The sea bream sex steroid profile is discussed in detail in chapter IV of this thesis.

I.4 Objectives

Early studies on fish sex steroids were predominantly carried out on species that first had major commercial importance such as the salmonids and the results obtained were frequently assumed to be typical of all teleosts. More recent studies, however, gradually indicated that salmonids are atypical teleosts in many respects and that there is a wide diversity in the nature of the steroid hormones produced in this group (Kime, 1993). Typical examples are many where hermaphrodite species in addition to the ignorance on the genetic basis of sexuality and the relative discrepancy on the species-specific steroid profiles, it is very difficult to link steroid plasma levels or metabolism to the sex inversion process since in the few studies carried out comparisons were made of the male and female stages reflecting the sex distinctive characteristics rather than the regulation of sex inversion itself (Baroiller *et al.*, 1999).

The main objective of this thesis is to correlate the steroid profile of the sea bream with the process of sex inversion and/or differentiation and generate further information to contribute to a general model to hermaphroditism in fish reproduction. To achieve this goal, sex steroid plasma profiles and steroidogenic capacities were followed in either estrogen-induced and naturally sex inverting sea bream. Hormonal sex reversal as been widely used as a tool for a better understanding of the mechanisms of sex differentiation and/or reversal in many teleost families (see review on hormonal induction of sex reversal in fish Pandian and Sheela, 1995). This strategy will allow to control differences in the sex inversion process due to hormonal exogenous interference making it possible to monitor in detail physiological and morphological changes in induced sex inverting specimens that are more difficult to obtain in natural sex inverting fish due to sea bream gonadal sex plasticity (Kadmon *et al.*, 1985). Very few studies on sex differentiation in hermaphroditic fish have been reported (Nakamura *et al.*, 1998). Specifically, in sea bream it is unknown when gonadogenesis occur and if the larval early stages are sensitive to hormonal treatments in order to differentiate directly as a female the same way adult sea bream can redirect gonadal sex from male to female (Happe and Zohar, 1988). Therefore time-phased estrogen

larval feeding scheme will be carried out in order to assess larval sensitiveness to estrogen encompassing gonadogenesis and gametogenesis in sea bream.

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CHAPTER II

THE EFFECT OF ESTROGEN ON THE GONADS AND ON *IN VITRO* CONVERSION OF ANDROSTENEDIONE TO TESTOSTERONE, 11-KETOTESTOSTERONE AND ESTRADIOL-17β IN *Sparus aurata* (TELEOSTEI, SPARIDAE)

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II The effect of estrogen on the gonads and on *in vitro* conversion of androstenedione to testosterone, 11-ketotestosterone and estradiol-17β in *Sparus aurata* (Teleostei, Sparidae)

II.1 Abstract

The effects of estrogen on gonad morphology and steroidogenesis of sea bream, *Sparus aurata*, a protandrous hermaphrodite teleost, were investigated. Fish were treated in winter/spring for different periods with 17α-ethynilestradiol ($ethE_2$; experiment 1) and in summer with two doses of E_2 (experiment 2). Estrogen was more effective in summer. Its main effect on the gonad was inhibition of testicular growth and of male germ cell development beyond the spermatogonia stage, including mitosis. The effect of estrogen on ovarian development was slight and only apparent at the end of experiment 2 in the higher dose group. Gonadal fragments were incubated at different times during treatment with androstenedione and the output of T, E_2 and 11KT were measured by radioimmunoassay. T and E_2 production were inversely correlated with the proportion of testicular tissue (and positively with ovarian tissue) in the gonad in experiment 2. However, the production of 11KT was not correlated with any type of tissue, possibly because of further metabolism. Inhibition of testicular development by estrogen was also associated with higher output of steroid conjugates.

KEY WORDS: sex reversal, feminisation, gonads, estradiol-17β, testosterone, 11-ketotestosterone

Running title: effect of estrogen on sea bream gonads

II.2 Introduction

Several endogenous and exogenous factors may trigger or mediate sexual inversion. Exogenous factors are mainly of social origin (Fishelson, 1970; Robertson, 1972; Fricke and Fricke, 1977; Reinboth, 1980; Cole and Robertson, 1988; Warner and Swearer, 1991; Sunobe and Nakazono, 1993). Sex ratios and hierarchical changes are perceived by the brain initiating a cascade that appears to involve neuropeptide Y (NPY, Kramer and Imbriano, 1997), GnRH (Grober and Bass, 1991; Kramer *et al.*, 1993) and GtH (Koulish and Kramer, 1989; Yeung *et al.*, 1993). Actions of GtH (and possibly GnRH) on sex reversal most likely occur through changes in steroidogenesis and steroid receptors in the gonads.

Steroids given at the appropriate time can influence sex differentiation both in gonochoristic (Yamamoto, 1958; Yamamoto and Kajishima, 1968; Badura and Friedman, 1988) and hermaphrodite species (Happe and Zohar, 1988; Chang *et al.*, 1995). Generally androgens induce masculinisation and estrogens feminisation. However, little is known of the short-term histological and steroidogenic changes that occur during natural and induced sex reversal. There are close relationships between developing male tissue and 11KT in blood plasma or its gonadal biosynthesis *in vitro* (Yeung and Chan, 1985; Nakamura *et al.*, 1989; Guiguen *et al.*, 1993; Guiguen *et al.*, 1995). Chang *et al.* (1995) found in black porgy, *Acanthopagrus schlegeli*, that, depending on the dose of E_2 used to induce sex change, testicular or ovarian tissue would develop and only 11KT, not T, correlated with testicular development. In the protandrous *Sparidentex hasta*, 11KT has also been suggested to be a reliable indicator for sex reversal or differentiation (Kime *et al.*, 1991). In the anemonefish, *Amphiprion melanopus*, 11KT was higher in males than females, while the opposite was true for E_2. During feminisation there is a reduction in 11KT and an increase in E_2 (Godwin and Thomas, 1993). Among other protandrous hermaphrodites, an unknown sterol is present in higher concentrations during the transition phase of sex reversal in sea bass, *Lates calcarifer* (Guiguen *et al.*, 1995). In protogynous species such as the grouper, *Epinephelus tauvina*, a shift toward the production of 11β-hydroxytestosterone (11βT) and 11KT occurs during masculinisation (Lee *et al.*, 1995).

The objective of the present work was to detail the histological and steroidogenic changes that result from estrogen-induced feminisation of the protandrous hermaphrodite sea bream, *Sparus aurata.* Previous studies have shown that sea bream can be readily feminised by E_2 (Reinboth, 1983; Happe and Zohar, 1988) and that 11-oxo androgens are produced by testicular tissue (Eckstein *et al.*, 1978). Plasma levels of E_2 were very low (108 $\pm$ 11 $pg.ml^{-1}$) in fish with ovaries containing mainly pre-vitellogenic oocytes, with highest levels (1669 $\pm$ 312 $pg.ml^{-1}$) found during the early vitellogenic phase (Kadmon *et al.*, 1985). In contrast, in fish with mainly testicular tissue highest levels were found when spermatogonia were mainly present (745 $\pm$ 142 $pg.ml^{-1}$, Kadmon *et al.*, 1985). Ovulatory females produce large quantities of 20β-reduced pregnanes (Canario *et al.*, 1995). No data on sea bream plasma androgens are available. In the present study sea bream were fed estrogen and changes in the gonadal status was assessed from their histology and from steroidogenic capacities. To overcome the problem presented by low endogenous steroids production, small fragments of gonads were incubated *in vitro* with androstenedione as precursor and the output of T, 11KT and E_2 measured.

11.3 Materials and Methods

Reagents - Hormones were purchased either from Sigma-Aldrich Co. (Poole, Dorset, UK) or Steraloids (Newport, RI, USA). *In vitro* culture reagents were purchased from Sigma-Aldrich Co. Hormone treatments were prepared by dissolving the steroid in ethanol, spraying the solution evenly onto the food pellets followed by evaporation in air.

Fish – Fish used in the experiments came from the same spawning stock and were raised in $3m^3$ tanks at the CIMSul experimental fish station (Instituto de Investigação das Pescas e do Mar, Olhão, Portugal) where the experiments were also carried out. Fish were placed in $1m^3$ round fibreglass tanks a week prior to starting the experiments and fed daily with 3% dry feed weight/wet fish weight of a commercial diet (Ewos Ltd,

Scotland). The tanks were supplied with well-aerated running seawater under natural photoperiod and temperature conditions.

Experiment I *(effect of 17α -ethynilestradiol)* – In a preliminary experiment, carried out between 23 June and 26 September 1991 (temperature range 22-28°C), 25 $mg.kg^{-1}$ diet of 17α-ethynilestradiol ($ethE_2$), a powerful estrogen agonist, was administered on alternate weeks to 150 g average weight sea bream. Treatment caused feminisation of the gonads (81.4 ± 3.3% ovarian tissue, compared with 14.3 ± 4.1% in controls, n=11) but it also caused a reduction in growth and food intake. In the actual experiment, to reduce the effect of $ethE_2$ on appetite, fish were treated with $ethE_2$ at a lower dosage and for different lengths of time. Forty fish, 40.7 ± 0.30g (mean ± SEM) were randomly placed in six tanks assigned to three duplicate experimental groups. Two of the groups were given a diet containing 15 $mg.kg^{-1}$ $ethE_2$ for 37 days (T1) or 112 days (T2). The third group (control; CTL1) was fed the same diet sprayed only with ethanol. Initially fish were treated daily with hormone but owing to their reduced appetite compared with controls, treatment with hormone was given every other day from day 24. Average weekly temperatures ranged between 12.8°C in December 1992, when the experiment started, to 19.7°C when it finished 22 weeks later (May 1993; Fig. 2.1).

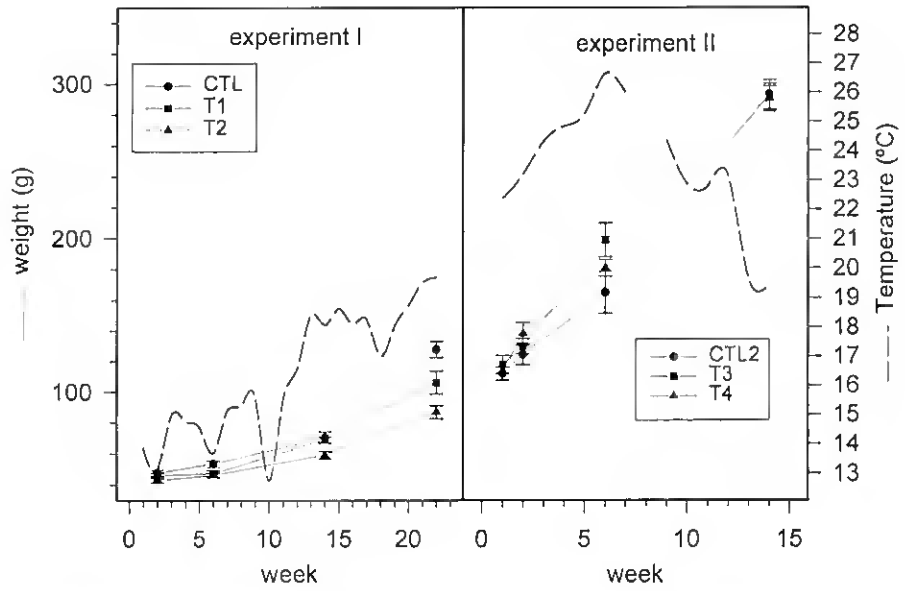

Figure 2.1 – Changes in weight and temperature in control and estrogen-treated fish in the two experiments. In experiment I there were no statistical differences in weight among groups until the 6th week. At week 14, CTL was significantly heavier than T2 but not T1 whereas by week 22 there were

significant differences between the three groups. In experiment 2, there was significant growth from weeks 2 to 14, but no difference between groups (two-way analysis of variance at $p<0.05$ level)

To follow the changes induced by treatment, samples of generally 12 fish per treatment were collected at weeks 2, 6, 14 and 22. Fish were anaesthetised with 2-phenoxyethanol, measured, weighed, bled, killed by decapitation and the gonads taken for histology and *in vitro* steroidogenesis.

Experiment II (effect of estradiol-17β) – To overcome the lack of appetite shown by fish treated with $ethE_2$ in the preliminary and in experiment I, in experiment II it was decided instead to treat the fish with E_2. To further improve appetite, cod liver oil (10 $ml.kg^{-1}$) was mixed in the diet of all groups. Twenty-seven fish from the same stock as the fish used in experiment I, mean weight 92.7 ± 0.98g, were randomly placed in six tanks assigned to three duplicate experimental groups. Two of the groups were treated with, respectively, 2 $mg.kg^{-1}$ (T3) and 15 $mg.kg^{-1}$ (T4) of E_2 in the diet. The third group acted as control and received the same diet sprayed only with ethanol (CTL2). The experiment lasted 14 weeks starting in June 1993 and finishing in October 1993. During this period average weekly temperatures ranged between 26.6 and 19.4°C (Fig. 2.1). The effect of treatment was followed by sampling 6 fish per treatment at 1, 2, 6 and 14 weeks (13 fish), as given above.

In vitro steroidogenesis - Fractions (5-10 mg) of gonads were incubated *in vitro* in Costar 12 well plates (Costar Corporation, Cambridge, USA) containing 1ml trout balanced salt solution (Jalabert and Fostier, 1984), 5 I.U. human chorionic gonadotrophin (hCG) to stimulate steroidogenesis and androstenedione (2 $\mu g.ml^{-1}$ in experiment I and 0.5 $\mu g.ml^{-1}$ in experiment II). No cofactors were used. Incubations were carried out for 48h at room temperature (21 ± 2°C) in a humid, oxygenated atmosphere and under constant gentle agitation. Incubates were stored at -20°C for later extraction and radioimmunoassay.

Steroid assays - Free, glucuronidated and sulphated fractions from blood plasma (100 µl) and *in vitro* incubates (1 ml) for radioimmunoassay were obtained using methodologies described by Scott and Canario (1992) and Canario and Scott (1989). Specificity tables for the E_2, T and 11KT radioimmunoassays (RIAs) used in the present

study have been previously published (Scott *et al.*, 1984). The E_2 RIA cross-reacted less than 0.1% with ethE_2. The limit of detection of the three radioimmunoassays was 200 pg/ml (blood plasma) and 40 pg.well^{-1} (incubates). Intra-assay and inter-assay precision (coefficient of variation) were, respectively, 7.5% and 12.4% for T, 8.2% and 11.6% for 11KT and 9.1% and 9.2% for E_2.

Histology - Gonads were fixed in Bouin's fixative for 36h, dehydrated and embedded in paraffin wax. Thick transverse sections (6 μm) were stained with Ehrlich's hematoxylin and eosin. Clear camera drawing and a polar planimeter were used to quantify areas occupied by testicular and ovarian tissues. The gonads were further classified on the basis of stage of maturity of germinal cells and the area occupied by each maturity stage (see Table 2.1). The gonadosomatic (GSI) index was calculated according to the formula:

$$\text{GSI}\,(\%) = \frac{\text{gonad weight}\ (\text{g})}{\text{fish total weight}\ (\text{g})} \text{x}100$$

To verify if there were differences in the proportion of testicular or ovarian tissue in different parts of the gonads, cross-sections from the anterior quarter, middle and posterior quarter of three gonads of each group from experiment II (week 14) were analysed by repeated measures one-way analysis of variance. Since the regions were statistically similar ($p=0.189$ for testicular tissue and $p=0.532$ for ovarian tissue), a cross-section posterior to the middle region was used to calculate testicular and ovarian indices as the product of GSI by the proportion of testicular or ovarian tissue. Parallel sections of tissue were used for the steroidogenic studies.

Statistics - Results are presented as mean ± SEM. The effect of treatment on growth, gonadal morphology and steroidogenesis was tested by two-way Analysis of Variance (ANOVA) followed by Tukey's Honestly Significant Difference test. Data was first log (weights and concentrations) or inverse sine (percentages) transformed before ANOVA. Plots in Figures are based on untransformed data. Statistical significance was considered at the 5% level.

Table 2.1 – Maturity scale used for staging testicular and ovarian regions of sea bream treated with estrogen.

Stage	Testicle	Ovary
0	Absence of, or a few scattered, male germ cells	Absence of, or a few scattered, female germ cells
I	Spermatogonia are the only germ cells present (<30% testicular area)	Exclusive, or almost exclusive, presence of primary germ cell nests
II	Spermatogonia are the only germ cells present (30-60% testicular area)	Primary germ cell nests predominate in relation to oogonia and exclusion of any other female cell
III	Spermatogonia are the only germ cells present (>60% testicular area)	Oogonia predominate in relation to oogonia mother cells nests and exclusion of any other female cell
IV	Presence of spermatocytes I	Pre-vitellogenic oocytes cover <50% ovarian area
V	Presence of spermatocytes II	Pre-vitellogenic oocytes cover 50-75% ovarian area
VI	Presence of spermatids	Pre-vitellogenic oocytes cover >75% ovarian area
VII	Presence of spermatozoa	
VIII	Spermatozoan cysts present (>10% testicular area) but no sperm duct formed (*vas deferens*)	
IX	Spermatozoan cysts of large dimensions and sperm duct present.	

II. 4 Results

II. 4.1 Effect of hormone treatment on gonadal morphology

Growth and gonadosomatic index - During experiment I, ethE_2-fed groups (T1 and T2) had reduced appetites and reduced growth ($p<0.001$) compared with control (CTL1; Fig. 2.1). When ethE_2 was replaced by E_2 (experiment II) a reduced appetite was not observed and growth was very similar among groups (Fig. 2.1). In experiment I the gonads were too small to obtain accurate GSIs. In experiment II, the initial GSI ranged

from $0.022 \pm 7.78 \times 10^{-5}$ to $0.030 \pm 6.16 \times 10^{-5}$ and it grew continuously throughout the experiment. However, the largest increase was in the control and T3 groups ($p<0.05$) after the 6th week (1.01 ± 0.019 and 0.77 ± 0.013). The ovarian and testicular portions of the gonad considered separately (Fig. 2.2), revealed that the testicular index was very significantly different between T4 and the other two groups at week 14 ($p<0.01$). In T4

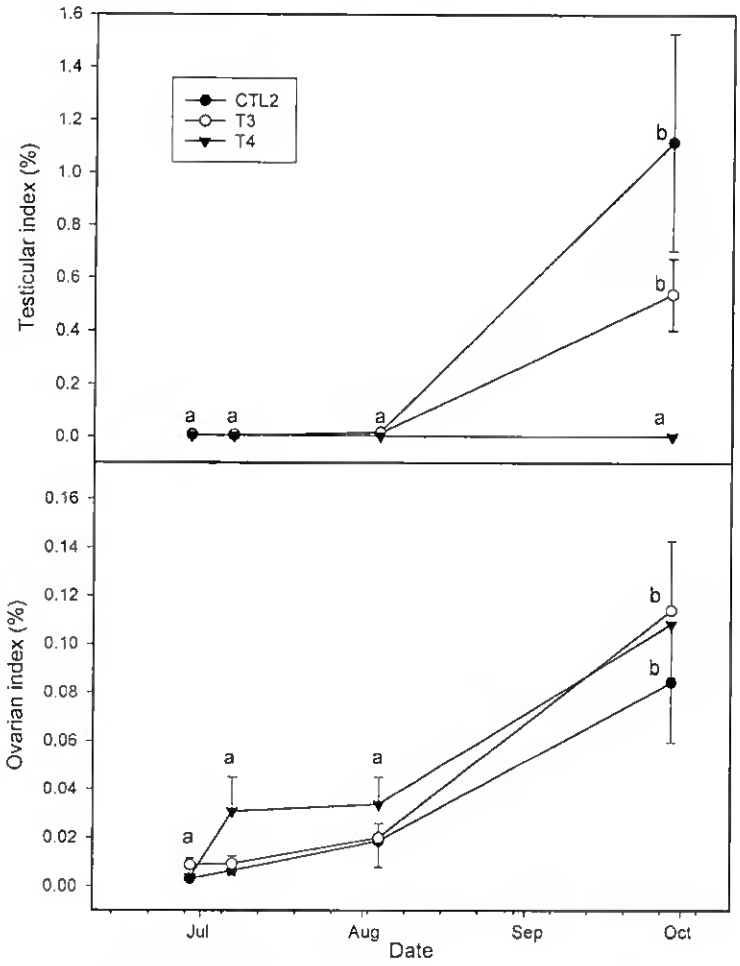

Figure 2.2 – Progression of testicular and ovarian indices in sea bream treated with E_2 (experiment 2). The two indices were calculated by multiplying the gonadosomatic index by the proportion of each type of tissue determined from histological sections. Different letters indicate statistically significant differences ($p<0.05$) among groups and sampling dates.

it was essentially unchanged during the experiment. From the 6th week there was an increase in the testicular index in T3 and CTL2 reaching $0.54 \pm 0.14\%$ and $1.12 \pm 0.41\%$, respectively, by the 14th week. In contrast, the ovarian index increased steadily in all groups until week 6 but there was a sharp increase beyond this period. At week 14, treated groups had an ovarian index slightly higher (0.11% in T3 and T4) than CTL2 (0.08%).

Effect of 17α-ethynilestradiol - Up to the 6th week the gonads in all groups contained essentially undifferentiated tissue (Fig. 2.3). By the 14th week control fish had more testicular ($45.7 \pm 7.7\%$) and less undifferentiated tissue ($48.2 \pm 8.48\%$) than

treated fish ($p<0.05$). Although there was a tendency for a slight decrease in testicular tissue in the control group between weeks 14 and 22, it was not significant ($p>0.05$). The proportion of testicular tissue in the treated groups was also virtually unchanged during this period as was the proportion of undifferentiated tissue in all groups ($p=0.93$). Treated fish had a higher proportion of undifferentiated tissue than control fish at both weeks 14 and 22 ($p=0.003$). No differences between groups in the proportion of female tissue were found ($p=0.384$) but there was an average increase of 32% for ovarian tissue in control and treated groups between weeks 14 and 22 ($p=0.009$), a period when hormone treatment had already ceased. No differences in the proportion of any type of tissue were ever found between the two levels of oestrogen treatment.

At week 14, 70% of control fish had spermatozoa but this proportion had decreased sharply by week 22. At this time most fish had only spermatogonia and spermatocytes I (Table 2.2). Greater development of testicular tissue was observed in the control group when compared with both treated groups at week 14 ($p=0.0004$). A significant difference with T2 was still evident at week 22 ($p=0.035$). Changes in testicular tissue were not so clear in T1 and T2. At week 14 most fish in T1 and T2 had a few spermatogonia covering less than 30% of testicular area. At week 22 less than 30% of the fish in T1 progressed to produce spermatids and spermatozoa, the remaining fish contained scarce spermatogonia or no visible male germ cells. In T2 none of the gonads progressed beyond spermatogonia.

In contrast to testicular tissue, ovarian tissue developed in a similar manner in all groups of fish during the whole experimental period. At week 14 the ovary consisted mainly of primordial germ cell nests or oogonia scattered throughout the ovary. At the end of the experiment 40% of all fish contained pre-vitellogenic oocytes in their ovarian tissue and no differences between groups were found ($p=0.57$). Only a thin layer of squamous follicular cells was present in pre-vitellogenic oocytes indicating that the follicles were not fully differentiated.

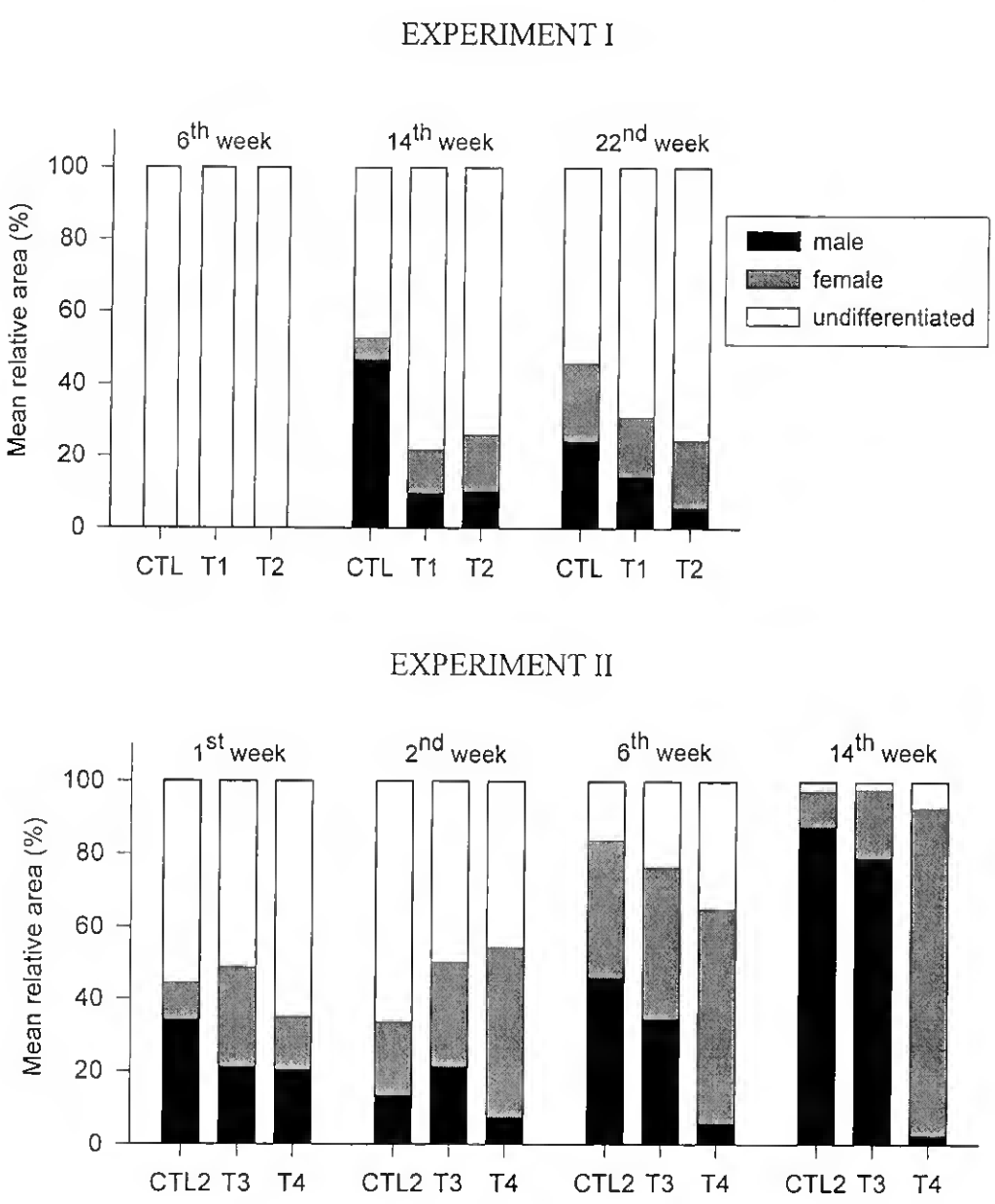

Figure 2.3 – Changes in the proportion of testicular, ovarian and undifferentiated tissues in the gonads of sea bream. Top: experiment I - fish were given a diet containing no estrogen (CTL1), 15 $mg.kg^{-1}$ $ethE_2$ for 37 (T1) or 112 days (T2). Control had a higher % of testicular and smaller undifferentiated area than treated groups only at week 14 ($p<0.05$). In contrast, treated groups had more undifferentiated tissue than control at weeks 14 and 22 ($P=0.003$). Bottom: experiment II - fish were given a diet containing no estrogen (CTL2), 2 $mg.kg^{-1}$ E_2 (T3) or 15 $mg.kg^{-1}$ E_2 (T4). There were no statistical differences in area occupied by ovarian or testicular tissue in CTL2 and T3 at any time during the experiment. T4 had a statistically significant lower proportion of testicular tissue than the other groups from week 6 and a higher proportion of ovarian tissue at week 14. See text for further details

Table 2.2 – Results from scoring maturity stages of testicular and ovarian tissue (for criteria see Table 2.1) in sea bream treated (T1 and T2) and not treated (CTL1) with ethE_2. Each dot (•) represents one fish.

Treatment	Week	Maturity Scale									
		0	I	II	III	IV	V	VI	VII	VIII	IX
Testes CTL1	14th				•	•			••••	•	
	22nd		••	•		•••			•	•	
Testes T1	14th	••	•••••								
	22nd	•••		••				•	•		
Testes T2	14th	•	•••••	•							
	22nd	••••	••	••							
Ovary CTL1	14th	••	••	••	•						
	22nd		•	•	••	•••	•				
Ovary T1	14th		•	•••	•••						
	22nd		••		•••	••					
Ovary T2	14th		•	•••	•••						
	22nd		••••		•	••	•				

Effect of Estradiol-17β -At the start of the experiment most of the gonads were filled with largely undifferentiated tissue (>50%), the remainder being divided almost equally between testicular and ovarian tissue (Fig. 2.3). At the end of the experiment the gonads of fish receiving the highest hormone dosage (T4) consisted mainly of ovarian tissue. In contrast, the gonads of the control group (CTL2) contained mainly testicular tissue (Fig. 2.4). There were no statistically significant differences in area occupied by ovarian or testicular tissue in the control and the lower dose E_2 groups. Significant differences were detected, however, in the proportion of testicular tissue between T4 and the other groups from week 6 of the experiment, and at week 14 for the proportion of

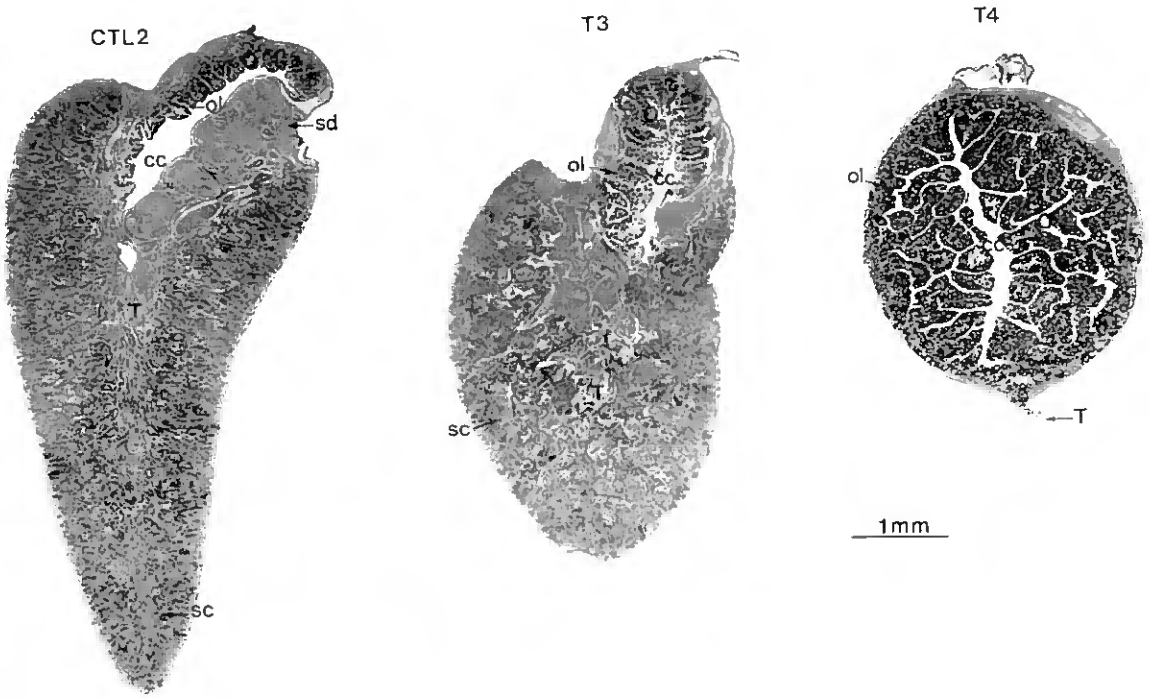

Figure 2.4 – Histological cross sections (H & E 6μm) of gonads from experiment II at week 14. CTL2: gonad from control fish showing a largely dominant mature testis filled with mainly spermatozoan cysts (sc) and a clearly formed sperm duct (sd). A small ovary (O) with ovarian lamella (ol) and central cavity (cc), is located dorsally. T3: gonad from a fish treated with 2 $mg.kg^{-1}$ E_2 showing less prominent testis and ovary. T4: gonad of fish treated with 15 $mg.kg^{-1}$ E_2 showing ovary and regressed testis.

ovarian tissue. There were no statistically significant differences in the proportion of undifferentiated tissue between the three groups ($p=0.202$). After the 2nd week and until the end of the experiment there was a progressive and significant reduction in undifferentiated tissue in all groups ($p<0.05$). Cytological observations also confirmed significant inhibition in testicular development in T4 six weeks after the beginning of E_2 treatment ($p=0.004$) and until the end of the experiment ($p<0.001$) (Table 2.3). Only one T4 fish had spermatocytes I at the first week and no stages beyond spermatogonia were observed after this period. In contrast, fish in CTL2 and T3 developed all types of male germ cells and at the end of the experiment they had functional testes filled with spermatozoa. The ovarian region in all groups showed a progressive change from a high proportion of oogonia and few pre-vitellogenic oocytes to a stage where pre-vitellogenic

Table 2.3- Results from scoring maturity stages of testicular and ovarian tissue (for criteria see Table 2.1) in sea bream treated (T3 and T4) and not treated (CTL2) with E_2. Each dot (•) represents one fish.

Treatment	Week	Maturity Scale									
		0	I	II	III	IV	V	VI	VII	VIII	IX
Testes CTL2	1			••	•		•			•	
	2		••	••							
	6						•	••		•	
	14										•••••• •••••
Testes T3	1		•	••		•		•			
	2		••	•	•						
	6			•	•		•	•	•	•	
	14									•	•••••• ••••
Testes T4	1	•	•	•	•						
	2		•••								
	6	••	••••								
	14	•••••• •••••	••								
Ovary CTL2	1			•	••	••					
	2		•		•	••					
	6					•••	•				
	14					••••••	•••••				
Ovary T3	1			•	•	•	••				
	2			•		••	•				
	6				•	•••	••				
	14					•••	•••••• ••				
Ovary T4	1			•	••	•					
	2				•		••				
	6				•	•	••	••			
	14					•	•••	•••••• •••			

oocytes covered ca 50% of the ovary (CTL2 and T2) and in some fish up to 75%. At week 14, 9 out of 13 fishes from T4 had more than 75% of ovarian section filled with pre-vitellogenic oocytes. This was greater than that observed in T3 and CTL2 (P=0.004) (T4; Table 2.3). There were no vitellogenic oocytes in any of the groups.

II.4.2 Steroid output by gonadal tissue

Effect of ethE$_2$ - Blood plasma steroid levels were very low and in a large number of samples below the detection limit of the radioimmunoassays (200 pg.ml^{-1}). For this reason they are not discussed.

In vitro incubates of transverse sections of gonads with androstenedione at week 14 and 22, contained mainly free T and 11KT (Fig. 2.5). However, at week 22 significant levels of glucuronides were also detected. No sulphates were detected in samples from either week 14 or 22.

The effect of ethE$_2$ treatment on 11KT production was only statistically significant in the glucuronide fraction at week 22. Glucuronide in T2 incubates was significantly higher than either T1 or CTL1, which contained similar levels. Production of free E$_2$ was significantly higher in T1 and T2 incubates than in control incubates at week 14. T2 contained significantly more free T than control incubates at week 14. No significant differences between any of the steroids were present at week 22. Indeed by week 22 the relative abundance of free and glucuronide of T or E$_2$ did not appear to be related to treatment, although, overall, more T and E$_2$ were present in T2 incubates than CTL1 or T1 incubates (Fig. 2.5). In all the fish studied more free E$_2$ was produced ($p<0.05$) than glucuronide. The proportion of the E$_2$ glucuronide fraction (45%) was higher than the glucuronides of androgens. Positive Pearson correlations ($r>0.60$, $n=22$) of total production (free + conjugated) between the three steroids were highly significant ($P<0.002$). None of the steroids produced was correlated to type of tissue.

Effect of E_2 - Blood plasma steroid levels were very low and were not considered to be meaningful. The production of all three steroids in the incubates decreased during the experiment ($P<0.001$; Fig. 2.6) and conjugates were very low with the exception of

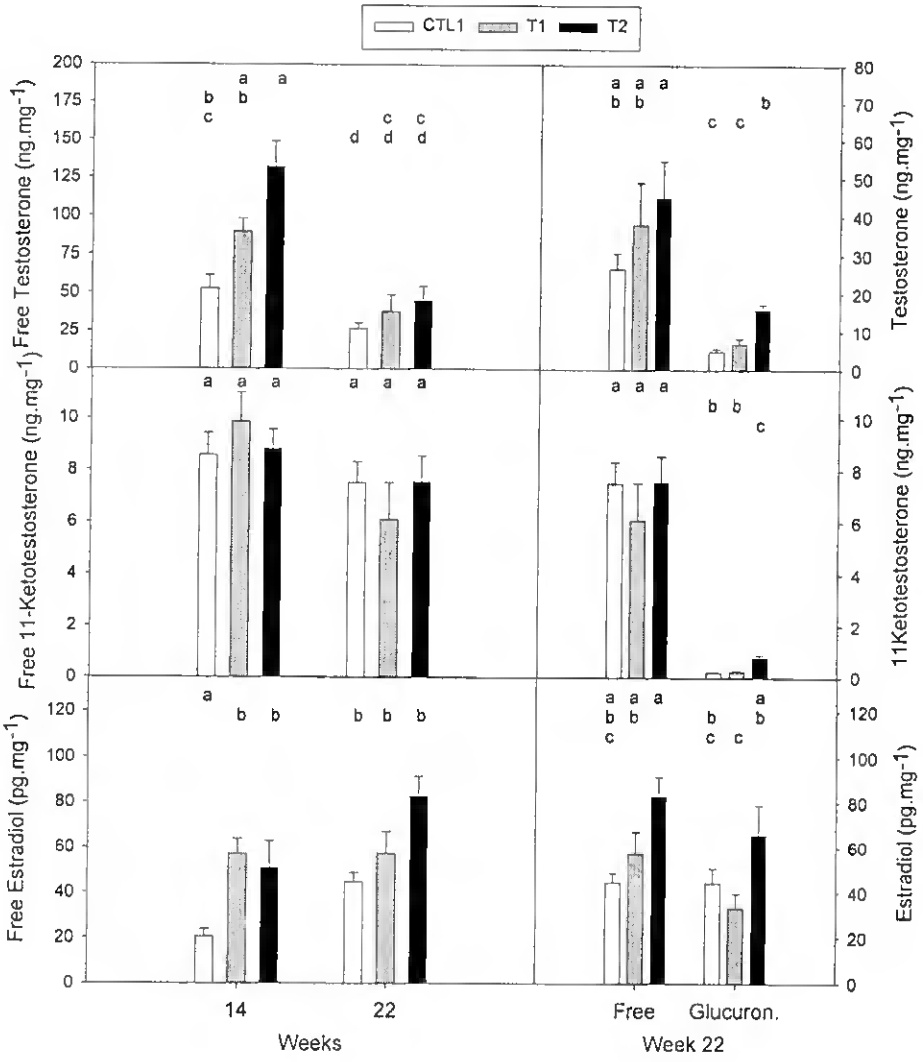

Figure 2.5 – Steroid output by gonadal tissue fragments incubated with androstenedione and measured by RIA in fish treated with ethE_2. Different letters above bars represent statistically significant differences ($p<0.05$) among groups and sampling dates on the left column and statistically significant differences among groups and form of steroid on the right column. Results are expressed per wet weight of tissue. Note differences of scale.

samples from week 14, which was carried out in October, at the onset of the period of natural spawning. Until the 6th week, no consistent significant differences between groups were noticed, but they were apparent for all steroids at week 14.

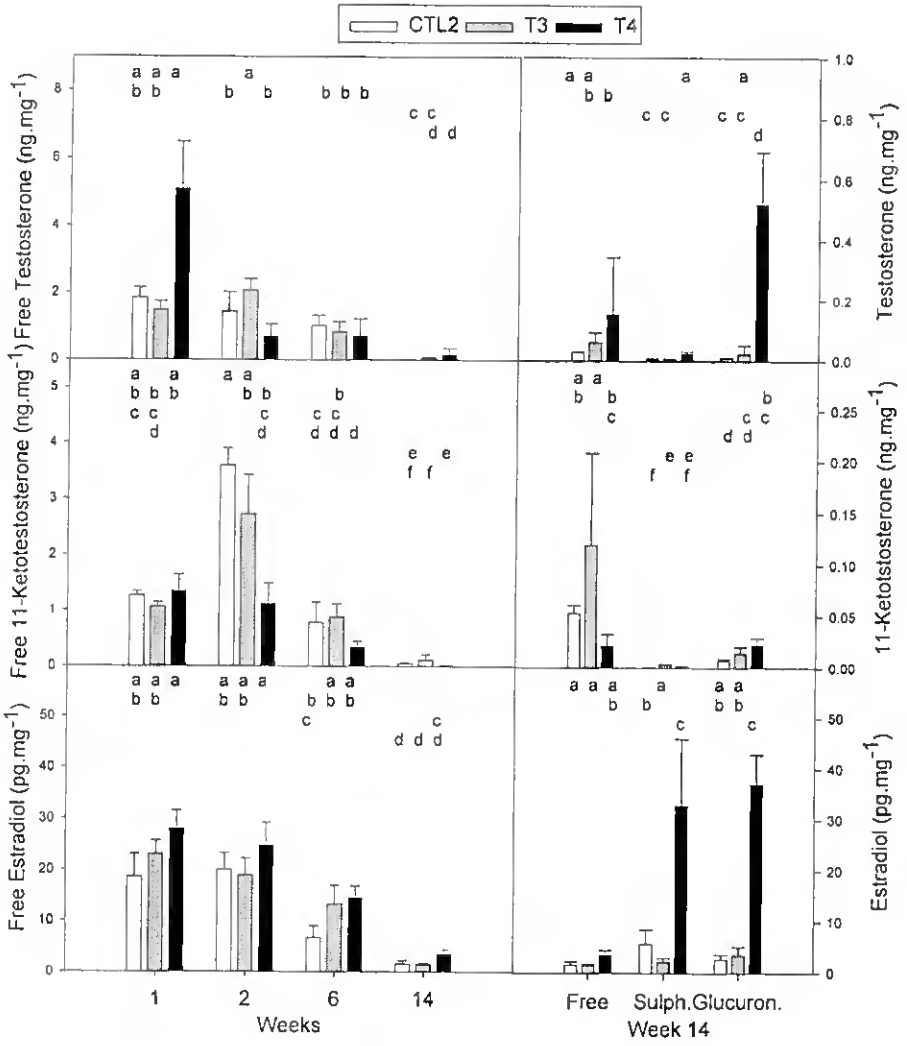

Figure 2.6 - Steroid output by gonadal tissue fragments incubated with androstenedione and measured by RIA in fish treated with E_2. Different letters above bars represent statistically significant differences ($p<0.05$) among groups and sampling dates on the left column and statistically significant differences among groups and form of steroid on the right column. Results are expressed per wet weight of tissue. Note differences of scale.

At the end of the experiment more total T was produced by T4 than by T3 or CTL2 ($p<0.05$), the difference between the latter groups was not significant. Sulphated T was only a small fraction compared to both free and glucuronidated T ($p<0.05$). There was a tendency for higher doses of E_2 treatment to cause increased production of glucuronides. Similar amounts of T sulphate and glucuronide were produced by CTL2, the main product being free steroid. T sulphate was present at the lowest concentration

in samples from T3 and there were no significant differences between the concentration of glucuronide and free steroid. There were statistically significant differences in production of T, its sulphate and glucuronide in T4. Glucuronide was present in the highest concentration followed by free and sulphate. Omission of the precursor (androstenedione) from gonadal incubates resulted in systematically low levels of T (9.2 $\pm$ 1.7 pg.mg^{-1} of in CTL2; 14.2 $\pm$ 3.1 pg.mg^{-1} in T3 and 22.4 $\pm$ 4.9 pg.mg^{-1} in T4).

The concentration of E_2 administered to fish influenced the production of 11KT and its metabolites. Overall, more 11KT and its metabolites were produced by T3, with no significant differences between CTL2 and T4. More free 11KT was produced followed by glucuronide and sulphate with significant differences between the three forms. T3 produced more free 11KT than T4 and more sulphated hormone than CTL2. No significant differences in production of the two forms of 11KT were found between T4 and CTL2. Glucuronide production was significantly higher in T4 than in CTL2. In the absence of precursor, total 11KT production was very low (CTL2= 6.07 $\pm$ 1.57 pg.mg^{-1}, T3= 12.2 $\pm$ 4.59 pg.mg^{-1}, T4=4.38 $\pm$ 0.98 pg/mg).

E_2 production followed a similar pattern to T production, although at levels 100 fold lower (Fig. 2.6). At week 14, more E_2 was produced by T4 ($p<0.05$) and mainly as sulphates and glucuronides. Without precursor (androstenedione), production of total E_2 was 1 fold lower (5.55 $\pm$ 1.51 pg.mg^{-1} in CTL2, 2.65 $\pm$ 1.16 pg.mg^{-1} in T3 and 8.08 $\pm$ 1.29 pg.mg^{-1} in T4). This contrasts with what was observed in androgen output, where omission of precursor caused a 2-3 fold reduction.

At the end of the experiment highly significant negative correlations were found between the proportion of testicular tissue in the incubation and T ($r<-0.65$, $p<0.001$, $n=23$) and E_2 ($r<-0.83$, $p<0.001$ $n=23$). The same steroids also showed highly significant positive correlations (albeit slightly lower) with the proportion of ovarian tissue. 11KT output was not correlated to any type of tissue ($r=0.04$, $p=0.843$ with ovary and $r=-0.36$, $p=0.871$ with testis, $n=23$ for both correlations).

II.5 Discussion

The main effect of estrogen on sea bream gonads was inhibition of testicular growth and inhibition of development of germ cells beyond spermatogonia, including mitosis. This effect was most likely, a result of direct action of estrogens on the testicular tissues. When the first experiment started, and up until at least 6 weeks, gonadal tissue was undifferentiated. By the 14^{th} week there was a significantly lower proportion of testicular tissue present in all the treated groups, even that which received only 5 weeks treatment. While control fish had testes with germ cells at all stages of spermatogenesis, in the treated groups spermatogenesis did not progress beyond spermatogonia. At the end of the experiment (6 weeks after T2 stopped receiving ethE_2) there was a gradation in the proportion of testicular tissue which was inversely related to the amount of estrogen treatment. A clear inhibition of spermatogenesis by estrogen was still noticeable with only 2 individuals in T1 and none in T2 having progressed beyond spermatogonia. In the second experiment, which started with about half of the gonadal volume already differentiated, estrogen administration caused a clear reduction in testicular tissue, which was noticeable after 6 weeks in the high dose group (T4). Although at the start of the experiment spermatogonia filled most of the testis, as the experiment progressed they were progressively reduced until few remained. In contrast a progressive development to spermiation was observed in control and low E_2 dose groups.

The effect of estrogen on ovarian development was very slight and was apparent only after 14 weeks in the second experiment. This was noticeable histologically by the predominance of pre-vitellogenic oocytes in the ovary. Thus, in the present study, the effect of estrogen is to increase the proportion of ovarian tissue at the expense of testicular tissue and the result is referred to as feminisation. This definition contrasts with that proposed by other authors who have considered feminisation the presence of a functional ovary (producing gametes) or clear inhibition of spermiation (Reinboth, 1962; Reinboth, 1983; Happe and Zohar, 1988; Chang and Lin, 1998). Measurements of E_2 and histological observations in the anemonefish *Amphiprion melanopus,* also

indicate that estrogen does not seem to influence the initiation of female function (Godwin and Thomas, 1993).

In the first experiment the gonads were so small that accurate weighing was not realistically possible. In the second experiment it was possible to show a natural increase in GSI. However, the GSI was larger in the groups with a dominant testis (CTL2 and T3). Recalculating GSI taking into consideration the proportion of testicle and ovary confirmed that significant alterations due to treatment occurred mainly in the testicular part of the gonad. Inhibition of spermiation and reduction in GSI by estrogen has also been observed in other hermaphroditic (Chang *et al.*, 1995) and gonochoristic species (Billard *et al.*, 1981; Jobling *et al.*, 1996; Christiansen *et al.*, 1998). It was not clear from light microscopy observations whether reabsorption of more advanced germ cells also occurred, but electron microscopy observations of E_2-treated eelpout (*Zoarces viviparous*) testis showed the presence of phagocytosed spermatozoa, possibly by Sertoli cells (Christiansen *et al.*, 1998) and this may also have happened in sea bream.

The smaller effect of ethE$_2$ (1st experiment) as compared to E_2 (2nd experiment) was most likely due to the presence of undifferentiated tissue for most of the period of T1 treatment, and part of T2. In July-August, when control fish developed mostly testicular tissue, treatment with ethE$_2$ (preliminary experiment) or E_2 (2nd experiment) gave similar results. Seasonal effects on the effectiveness of steroid treatment are relevant since there seems to be natural changes favouring the development of an ovary (increase in the proportion of ovarian tissue in all groups between weeks 14 and 22 in first experiment) or a testis (control group in second experiment). The effect of ethE$_2$ on appetite (and growth) has not been reported before and suggests care may be required with the methodology of feeding steroids to fish.

Since most fish had extremely low steroid levels in blood plasma which were mostly undetectable by the assays used in the study, the steroidogenic activity of the gonads was estimated on the basis of conversion of androstenedione to E_2, T and 11KT. This methodology has also been used in studies of steroidogenesis in other species such as the protogynous wrasse (*Thalassoma duperrey*; Nakamura *et al.*, 1989). However, although results from this type of study can provide an indication of steroidogenic

activity they should be considered with caution, since the relatively large mass of exogenous steroid used can affect enzyme activity (Abdullah and Kime, 1994). For example, the amount of precursor used was reduced in the second experiment by 25% and the reduction of measured output decreased 90% for T, 25% for 11KT and 50% for E_2. Another problem with this approach is the possible production of non-immunoreactive metabolites. An attempt was made to estimate the presence of non-immunoreactive steroids by measuring unmetabolised precursor, but there was a significant amount of non-parallel cross-reaction with the androstenedione RIA (characterised by Scott *et al.*, 1984). The presence of 2 immunoactive peaks other than androstenedione was confirmed by thin-layer chromatography (data not shown).

Despite these limitations, the results indicated that T and E_2 were largely associated with feminisation of the gonads. In the first experiment more free T and E_2 was produced at week 14 when significant differences in the proportion of testicular tissue were observed. At week 22 overall production (free + glucuronide) of the two steroids was also higher in T2. Similarly, at the end of the second experiment, T4, the group in which clear feminisation of the gonad occurred, showed higher production of T and E_2 compared with other groups. This relationship was also observed in control tissues incubated without precursor. Furthermore there were clear negative correlations between the proportion of testicular tissue in the gonad and output of E_2 and T (and positively with ovarian tissue). This suggests that the ovarian elements are responsible for the conversion of available androstenedione to T and E_2, which therefore contain 17β -hydroxysteroid dehydrogenase (17β-HSD). The enzyme has also been found to be more active in ovary of the protandrous *Pagellus acarne* (Reinboth *et al.*, 1986) and *Rhabdosargus sarba* (Yeung and Chan, 1985). Given the high output of the 2 androgens, it is assumed that 17β-HSD was not limiting for their production. However, the specificity of steroidogenic enzymes has not been studied in fish and it is possible that more than one form of gonadal 17β-HSD exists, one with a preference for androgens as substrate, the other for estrogens (Andersson, 1995; Poutanen *et al.*, 1995). If this was the case, and providing aromatase activity was not limited, estrone, which we did not measure, could have accumulated due to low activity of "estrogenic 17β-HSD". However, considering the incipient development of follicular cells observed in the histological preparations, the most likely possibility is that aromatase, which is present

in the granulosa cells (Young *et al.*, 1983; Kagawa *et al.*, 1984), was the main limiting factor in E_2 production. A low aromatase activity leading to estrone synthesis was found in the pre-vitellogenic ovary of a protandrous sea bass (Guiguen *et al.*, 1993). Unlike in the protandrous black porgy, *Acanthopagrus schlegeli* (Chang and Lin, 1998) no noticeable stimulation of aromatase activity by E_2 was observed in this study.

11-Oxygenated androgens, including 11KT, 11β-hydroxyandrostenedione and 11βT are very powerful androgens and markers of male phenotype (Kime *et al.*, 1991; Guiguen *et al.*, 1995). However, androgenicity is probably markedly reduced by metabolism. Conversion to 11KT was very high in sea bream, indicating very high activities of 11β-hydroxylase and 11β-hydroxysteroid dehydrogenase. Because of the lack of correlation between 11KT and any type of tissue one speculation is that the androgens were being further metabolised (possibly through A ring reduction) by enzymes that were present in more than one type of tissue. This is also suggested by the clear decline in output in all immunoreactive steroids as the second experiment progressed. In support for this hypothesis Guiguen *et al.* (1995) found 5β-reductase to be present in ovary, testis and transitional gonads of the protandrous sea bass, *Lates calcarifer*. The same authors also found a shift from the production of 11-oxygenated androgens to an estrogen-like ester metabolite in "late transitional gonads".

There was an association between conjugate production and the decrease in testicular development more clearly seen in the second experiment. This could also be related to a mechanism limiting 11KT production. 11KT is a mitogenic hormone responsible for proliferation of spermatogonia (Miura *et al.*, 1996) and any limitation in its availability would significantly decrease the androgen/estrogen ratio facilitating feminisation. Steroid conjugates, being very water soluble, would rapidly be removed from the gonad through the blood stream.

GtH, GnRH and NPY have been effective in causing sex change in hermaphrodite species, possibly through a common mechanism involving gonadotrophin action on the gonad, on follicle development and on steroidogenesis (Koulish and Kramer, 1989; Yeung *et al.*, 1993; Kramer and Imbriano, 1997). Follicular aromatase is required for estrogen production leading to feminisation and is probably activated by gonadotrophin

(Dépêche, 1981; Kagawa *et al.*, 1984). However, the receptors acted on by estrogen are already present in the testicular tissue. Recently cDNA for two estrogen receptors have been cloned in sea bream, one of them, homologous to mammalian estrogen receptor β, being most abundant in the testis and ovary (Socorro *et al.*, in press). Further information on the location, control of steroidogenic enzyme activity and estrogen receptor expression is essential to unravel the mechanisms of sex reversal in hermaphrodite fish.

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CHAPTER III

GONADAL STEROIDOGENESIS IN RESPONSE TO ESTRADIOL-17β ADMINISTRATION IN THE SEA BREAM (*Sparus aurata* L.)

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III Gonadal steroidogenesis in response to estradiol-17β administration in the sea bream (*Sparus aurata* L.)

III.1 Abstract

The sea bream (*Sparus aurata*) is a protandrous hermaphrodite teleost fish in which estrogen administration induces testicular regression without influencing ovarian development. To analyse the changes in steroidogenesis of estrogen-treated and untreated fish, fragments of gonads were incubated with tritiated 17α-hydroxyprogesterone and the metabolites identified. The amount of recovered radioactivity decreased with incubation time and was lower in gonads containing a larger proportion of ovarian tissue. The difference in steroidogenic capacity between control and estrogen-treated groups was generally quantitative rather than qualitative and paralleled the observed histological changes. The same metabolites were identified in all three groups but estrogen treatment caused a marked inhibition of 5β-reduction, 3α-reduction, side-chain cleavage and 11β-hydroxylation. The main androgens identified were 11β-hydroxy-4-androstene-3,17-dione and 3α-hydroxy-5β-androstan-3,17-dione, both inhibited by estrogen treatment. Of the more polar pregnanes, 3α,17,20α-trihydroxy-5β-pregnane and 3α,17,20β-trihydroxy-5β-pregnane were detected in significant amounts but only the latter appeared to be associated with development of the testis (in the untreated fish). A feature of sea bream gonadal steroidogenesis less common in teleosts was the presence of 6α- and 6β-hydroxylation, the latter appearing to be stimulated by estrogen treatment. The role of many of the identified metabolites in teleost reproduction remains unknown.

KEYWORDS: steroidogenesis, estrogen, Sparus aurata, teleost

Running title: steroidogenesis in sea bream

III.2 Introduction

It is well established that in vertebrates gonadotrophins regulate gametogenesis by acting directly on germ cells and also indirectly by stimulating of steroid hormone secretion (Nagahama, 1994). In fish, early studies of steroid biosynthesis focused mainly on salmonids, where E_2[(1)], T and 11KT or 17,20βP predominate in the reproductive cycle. Females often show rising levels of E_2 and T during vitellogenesis and sharp increases of 17,20βP during ovulation, while males secrete androgens as T and 11KT during early spermatogenesis and progestagens as 17,20βP seem to play a role in final gamete maturation. The measurement of only these steroids in many species, however, can be misleading since blood levels are frequently low and there are a variety of alternative steroid biosynthetic pathways (Scott and Canario, 1987; Kime, 1993). *In vitro* incubations with radiolabelled precursors have been used to identify the steroidogenic capacity of the gonad to produce steroids with possible functions on gamete maturation, sex differentiation and sex inversion (Kime and Groves, 1986; Schoonen *et al.*, 1988; Kime *et al.*, 1991b; Guiguen *et al.*, 1995; Ponthier *et al.*, 1998). However, a common pattern correlating sexual status and steroid output has been difficult to establish, especially in hermaphrodite species. For example, in the hermaphrodites, *Pagellus acarne*, *Pagellus erythrinus*, *Serranus cabrilla* and *Diplodus sargus*, 11βT was suggested to be the main androgen and 11KT was not detected (Idler *et al.*, 1976). However, in another hermaphrodite fish, *Sparidentex hasta*, 11KT was considered a reliable indicator of the occurrence of sex reversal (Kime *et al.*, 1991a). Whether these correspond to real species differences or are the result of different experimental methods and/or insufficient data requires clarification.

Existing studies on the steroidogenic capacity of the gonads of the protandrous hermaphrodite sea bream, *Sparus aurata*, are scarce and incomplete. The metabolic products of [^{14}C]pregnenolone (Colombo *et al.*, 1972) were reported to be diverse in mature gonads, but relatively few were positively identified. During sex inversion, testis and ovary

(1) See Table 1 for steroid names and abbreviations

have been reported to produce mainly P and 17P (and androstenedione in the case of the testis). When [^{3}H]androstenedione was used as precursor in 2-year old sea bream gonads, Eckstein *et al* (1978) only identified T, 11KT, and 11βT but reported an additional unidentified compound in "considerable" quantities. In a recent study we have shown that the ability of the gonad *in vitro* to produce E_2 and T from androstenedione in sea bream treated with estrogen inversely correlated with the proportion of testicular tissue, while production of 11KT was not correlated to any type of tissue, possibly because it underwent further metabolism (Condeça and Canario, 1999). The main effect of estrogen on sea bream gonads was the inhibition of spermatogenesis rather than stimulation of ovarian development, and testicular inhibition was also associated with a higher output of steroid conjugates. The concentration of these steroids in blood plasma of most fish were too low for detection. Altogether this points to the requirement of a better understanding of the steroidogenic potential of sea bream gonads . The objective of this work, therefore, was to further elucidate the main steroidogenic pathways in the gonads of sea bream, with particular emphasis on those associated with gonadal sex inversion. For this purpose products from *in vitro* incubations of gonadal fragments with radioactive and non-radioactive precursors were analysed from control and estrogen-treated sea bream.

III.3 Material and Methods

Reagents - Hormones were purchased either from Sigma-Aldrich Co. (Poole, Dorset, UK) or Steraloids (Newport, RI, USA). 3α,20β-Hydroxysteroid dehydrogenase (3α,20β-HSD), cofactors and i*n vitro* culture reagents were purchased from Sigma Sigma-Aldrich Co. The steroid 6αA was synthesized from 6α,17,21P by oxidation with sodium bismuthate (Bush, 1961).

Fish and experimental design- The fish and basic experimental procedures were those described by Condeça and Canario (1999). In short, twenty-seven fish, mean weight 92.7± 0.98g, were randomly placed in six tanks assigned to three duplicate experimental groups. Two of the groups were treated with, respectively, 2 mg kg^{-1} (T3) and 15 mg kg^{-1} (T4) of E_2

in the diet. The third duplicate group acted as control and received the same diet sprayed only with ethanol (CTL2). The experiment lasted 14 weeks starting in June 1993 and finishing in October 1993. Steroidogenesis was studied by sampling 2 fish per treatment at 1, 6 and 14 weeks (4 fish/ treatment) using the methodology given below. The main effect of estrogen on the gonad was the inhibition of testicular growth and of male germ cell development beyond the spermatogonia stage, including mitosis (Condeça and Canario, 1999). At the end of the experiment (week 14) the relative size of the testes (testicular index) was higher in CTL2 and T3 as compared to T4. In contrast, the relative size of the ovary (ovarian index) was not significantly affected by treatment. To confirm the identity of some of the radioactive products by gas chromatography and mass spectrometry (GC-MS) analysis further incubations were carried out at a later stage with untreated fish using unlabelled 17P.

In vitro steroidogenesis – Transverse sections (10-20 mg) of the gonads were incubated in Costar 12 well plates (Costar Corporation, Cambridge, USA) containing 1ml trout balanced salt solution (Jalabert and Fostier, 1984) and 0.6-1.2 μCi of radiolabeled [1,2,6,7-^{3}H]17P (93Ci/mmol specific activity; Amersham Life Science, Buckinghamshire, England). Incubations were carried out at room temperature (21±2°C) in a humid and oxygenated atmosphere with constant gentle agitation. Aliquots (200 μl) of incubation media were removed after 1, 2, 4 and 18.5 hours, frozen in liquid nitrogen and stored at -20°C for later extraction.

Steroid extraction - Free, sulphate or glucuronide fractions of steroids from each incubate were separately extracted twice with 4ml of diethyl ether using the methods described in Scott and Canario (1992) and Canario and Scott (1989a). Recoveries were estimated by counting 10 μl from aliquots before and after extraction. Radioactivity was measured in a Beckman L60000IC scintillation counter (Beckman Instruments Inc., Fullerton, U.S.A.).

Table I – List of steroid abbreviations and systematic names of steroids mentioned in the text. Listed are also the steroids identified. Identity of metabolites marked with (1) was confirmed by recrystallization to constant specific activity with authentic steroid in different solvent systems and with (2) was confirmed by GC-MS.

Steroid abbreviation	Systematic name	Identification		
		Positive	Tentative	Suggestive
A	4-androstene-3,17-dione		✓	
11βA	11β-hydroxy-4-androstene-3,17-dione	✓(2)		
11KT	17β-hydroxy-4-androstene-3,11-dione		✓	
A5β	5β-androstane-3,17-dione			✓
3αA5β	3α-hydroxy-5β-androstan-17-one	✓(1)		
17,20αP	17α,20α-dihydroxy-4-pregnen-3-one		✓	
17,20βP	17α,20β-dihydroxy-4-pregnen-3-one		✓	
6α,17P	6α,17α-dihydroxy-4-pregnene-3,20-dione		✓	
6β,17P	6β,17α-dihydroxy-4-pregnene-3,20-dione		✓	
17P5β	17α-hydroxy-5β-pregnane-3,20-dione		✓	
3α,17P5β	3α,17α-dihydroxy-5β-pregnan-20-one	✓(1)		
3β,17P5β	3β,17α-dihydroxy-5β-pregnan-20-one		✓	
17,20αP5β	17α,20α-dihydroxy-5β-pregnan-3-one			✓
17,20βP5β	17α,20β-dihydroxy-5β-pregnan-3-one		✓	
3α,17,20αP5β	3α17α,20α-trihydroxy-5β-pregnane	✓(2)		
3α,17,20βP5β	3α17α,20β-trihydroxy-5β-pregnane	✓(2)		
3α,6α,17α,20αP5β	3α,6α,17α,20α-tetrahydroxy-5β-pregnane	✓(2)*		
E_2	3,17β- dihydroxy -1,3,5(10)-estratrien			
Estrone	3-hydroxy-3,5,10-estratrien-17-one			
T	17β-hydroxy-4-androsten-3-one			
Adrenosterone	4-androstene-3,11,17-trione			
11βT	11β,17β-dihydroxy-4-androsten-3-one			
A-3,6,17-trione	4-androstene-3,6,17-trione			
6αA	6α-hydroxy-4-androstene-3,17-dione			
6βA	6β-hydroxy-4-androstene-3,17-dione			
6β,17βA	6β,17β-dihydroxy-4-androsten-3-one			
17βA5β	17β-hydroxy-5β-androstan-3-one			
3α,17βA5β	3α,17β-dihydroxy-5β-androstane			

3β,17βA5β	3β,17β-dihydroxy-5β-androstane
A5α	5α-androstane-3,17-dione
Progesterone; P	4-pregnene-3,20-dione
17P	17α-hydroxy-4-pregnene-3,20-dione
17,21P	17α,21-dihydroxy-4-pregnene-3,20-dione
17,20β21P	17α,20β,21-trihydroxy-4-pregnen-3-one
Cortisol	11β,17α,21-trihydroxy-4-pregnene-3,20-dione
6α,17,21P	6α,17α,21-trihydroxy-4-pregnen-3,20-dione
17,21P5β	17α,21-dihydroxy-5β-pregnan-3,20-dione
3α,17,21P5β	3α17α,21-trihydroxy-5β-pregnan-20-one
3α,6α,17P5β	3α,6α,17α-trihydroxy-5β-pregnan-20-one

* Steroid identified from incubation with cold 17P only

Steroid separation – Steroid metabolites were separated sequentially by reverse phase thin layer chromatography (RPTLC) and normal phase thin layer chromatography (TLC) as follows:

I – Samples were first separated for 90 min on Whatman LKC18F silica plates (Whatman Inc. New Jersey, USA) with methanol:water (80:20) as mobile phase and the following cold steroid standards were used: cortisol; 17,20αP; 17,20βP; 17,21P; 17,20β21P; 17P; P; T; A; 11βA; 11βT; 11KT; E_2; 3α,17,20αP5β; 3α17P5β; 17P5β; 3αA5β. Radioactivity was scanned with a radiochromatograph Bioscan (Lablogic, Sheffield, England). Lanes were divided in 0.5cm fractions, scraped off and steroids eluted with pure ethanol (3X1ml) which was subsequently evaporated under nitrogen.

II - Each fraction was then applied and run on normal phase thin-layer chromatography (TLC; Whatman LK6DF silica gel 60Å plates) for 50 min with chloroform:methanol (48:2) as the mobile phase. Steroid standards were the same as those indicated for RPTLC. With the chromatograms from the two runs it was possible to build a composite bidimensional chromatogram using the coordinates of the TLC and RPTLC in each axis, which allowed the isolation and quantification of most individual products.

Peaks were scrapped off and eluted with dichloromethane:ethanol (80:20) and stored in absolute ethanol for identification.

Steroid identification – Steroids were initially tentatively identified on TLC by isopolarity with cold standards before and after microchemical and/or enzymatic reactions. The identity of some radioactive products was confirmed by recrystallization to constant specific activity with authentic steroid in different solvent systems (Axelrod *et al.*, 1965). Other steroids, either because they were difficult to obtain, or because of their cost were identified by GC-MS from incubations of gonads with unlabelled 17P (see table I). Identifications were rated as suggestive, tentative, or positive largely on the basis of the classification system proposed by Sandor and Idler (1972).

The following solvent systems were used as mobile phase for TLC - I-IV chloroform: methanol (respectively, 47.5:2.5; 48:2; 49:1 and 49.5:0.5), V chloroform, VI dichloromethane: diethyl ether (50:20) – and RPTLC - VII methanol: distilled water (80:20). Steroids with a double bond between C4 and C5 (4-ene steroids) were detected by UV light (254nm). 5(α,β)-Reduced steroids with hydroxyl groups were detected by immersion of plates in 5% (w/v) phosphomolybdic acid/ethanol followed by heating to 100°C for up to 15 min. 5(α,β)-Reduced steroids with mostly ketone groups were sprayed with a solution of freshly made benzyltrimethylammonium hydroxide (Fluka Chemie AG, Buchs, Switzerland): 0.5% n-dinitrobenzene in distilled ethanol (1:2). Microchemical reactions employed were largely based on the protocols of Bush (1961) with modifications described by Canario and Scott (1989b) and Kime (1978). It should be noted however that, contrary to earlier views (Bush, 1961; Norymberski, 1967), periodate oxidation cannot be used to diagnose $C_{17,20}$- dihydroxy steroids, since it will also oxidize 17-hydroxy-20-ketones to 17-ketosteroids (Lee *et al.*, 1998). We have confirmed this observation and verified that periodate also oxidizes overnight to 6-oxy-17-hydroxypregnanes such as 3α,6α,17P5β.

Trimethylsilyl (TMS) and oxime-trimethylsilyl (OTMS) derivatives for GC-MS were prepared by mixing the metabolites with methoxyamine hydrochloride (2.5mg) in pyridine

(50µl) and allowed to react for 1h at 100°C. Pyridine was removed by evaporation under N_2 and trymethylsilylimidazole (100µl) was added. The sylilation was conducted at 100°C for 1h. Steroid derivatives were injected (splitless mode at 250°C) in a Varian GC-MS (GC: Star 3400cx; MS: Saturn3; Varian chromatography systems, U.S.A.) fitted with a 15-meter DB5 non-polar fused silica capillary column. After a 1min delay for attaining the starting the temperature (160°C), a gradient was set to 190°C at 15°C/min, then to 260°C at 2°C/min, held for 10 min and finally to a maximal temperature of 300°C at 10°C/min. Mass spectral data were acquired by scanning the 55-650 mass range. The GC-MS identification criteria were correct retention time and simultaneous occurrence of minimum pre-defined values for 3 different spectra comparison-similarity indexes – fit, reverse fit, and purity - at respectively 900, 600 and 600. To calculate the indexes, both target (sample) and library (steroid standard) mass peaks were replaced by the square root of the product of the mass by the intensity and then reduced, respectively, to the highest 16 and 8 mass peaks (to avoid characteristic large mass peak elimination). Fit was calculated as follows:

$$Fit = 1000 * \frac{(\sum_{m=1}^{k} Tm * Lm)^2}{\sum_{m=1}^{k} Tm^2 * \sum_{m=1}^{k} Lm^2}$$ where Lm is the square root of the product of the mass by the intensity at mass m in the reduced reference library spectrum while Tm is the product of the mass by the intensity at mass m in the reduced target spectrum. Reverse fit is calculated in the same way as fit but considering only masses in the reduced target mass spectrum. For the calculation of purity all masses are considered. The maximum value of 1000, according to the index used, indicates best fit or highest purity.

Metabolite yields– Metabolite yields were expressed as percentage of total radioactivity added to each incubation well. Because of loss of material, recoveries for the incubates carried out with gonads from the first week could not be established and for practical purposes were assumed to be the same as the average for each group in the 4th week. Relative enzymatic activity was calculated as the percentage yield of steroids metabolised by a particular enzyme.

III.4 Results

III.4.1 Recovery of radioactivity

The recovery of radioactivity was not constant throughout the experiment. The amount of recovered radioactivity decreased with incubation time and varied according to the type of incubated tissue and treatment. At week 14, 18.5 hours of incubation of group T4, containing mainly ovarian tissue (>90%), led to a recovery of radioactivity of just over 50%, compared to 70% and 80 % in T3 (containing 20% ovarian tissue and 78% testicular tissue) and control (containing 15% ovarian tissue and 85% testicular tissue), respectively. Separate incubations of ovarian and testicular tissue from group T3 at week 14 confirmed that more unidentified water-soluble compounds (smaller recoveries) were being obtained with the ovarian tissue than with the testicular tissue (Fig. 1).

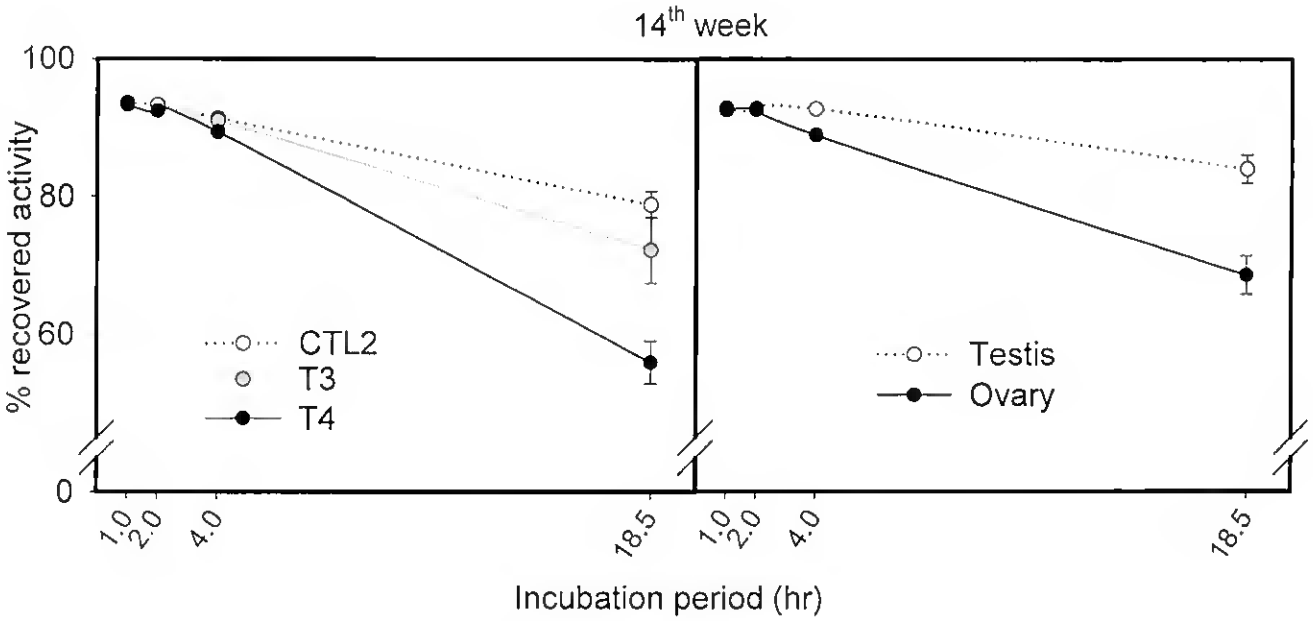

Figure 3.1- Percentage of radioactivity (free +conjugated metabolites) recovered from incubates of sea bream gonads with [^{3}H]17P as a function of duration of incubation. Left panel) radioactivity recovered from gonads of fish administered 2 mg.kg^{-1} (T3), 15 mg.kg^{-1} (T4) E_2 and no E_2 (CTL2) in food for 14 weeks. Right panel) Radioactivity recovered from separate testicular and ovarian portions dissected from T3 fish (n=2). Note that recovery decreased with time and was lower in ovary or in gonads with a predominance of ovarian tissue (T4).

In the recovered radioactivity, free metabolites were generally more abundant, except for longer incubation periods (18.5 hours) and towards the end of the experiment (week 14), when glucuronides predominated. Sulphated metabolites were generally low. No relationship was found between treatment and sulphate or glucuronide production.

III.4.2 Steroids Identified

Three regions with overlapping peaks were obtained on RPTLC. Region RF1, with higher retention time (RF < 0.30), yielded 9 metabolites on normal phase TLC (named A to I in order of decreasing polarity). Oxidation of A and B with periodate yielded a compound isopolar with 3αA5β (system IV) but not with any of the 5α/β-reduced epimers. Metabolite B was oxidised by 3α,20β-hydroxysteroid dehydrogenase (3α,20β-HSD) with NAD^+ into a compound isopolar with 17P5β. On system I, metabolite A was isopolar with 3α,17,20αP5β and metabolite B with its 20β isomer, but not 3α,17,21P5β, and were identified as such. Metabolites C, G and H when oxidised with chromium trioxide yielded compounds isopolar with A5β but not A5α or androstenedione (system V). On system II, C was isopolar with 3α,17P5β and 3α,17βA5β. Acetylation yielded a product isopolar with 3α,17P5β. Metabolite G and H were isopolar with, respectively, 3αA5β and 17P5β. Acetylation of G gave a product isopolar with the acetate of 3αA5β and H did not react. C and G were recrystallized to constant specific activity with, respectively, 3α,17P5β and 3αA5β. H was identified as 17P5β. Metabolite D was isopolar with 17,20αP5β both on TLC and RPTLC. The low yield of this metabolite prevented further characterisation. Metabolite E was isopolar with 1720βP5β on TLC and RPTLC and yielded a compound isopolar (system IV) with A5β after periodate oxidation. Since no other 5β-pregnane-3-one (including 17,20(α,β),21P5β,17,21P5β or 17P5β) had the chromatographic behaviour of D and E, they were identified respectively as 17,20αP5β and 17,20βP5β. Metabolite F was isopolar with 3β,17βA5β and 3β,17P5β in system II. It reacted with periodate originating a compound isopolar with 3βA5β. Borohydride reduction of F and 3β,17P5β gave isopolar products, resulting from reduction of the 20-ketone, and confirmed its identity as 3β17P5β.

Metabolite I had the mobility of A5β and its product of borohydride reduction was isopolar with 3α,17βA5β. Metabolite I was, thus, identified as A5β.

Region RF2 (RF 0.30-0.43) yielded 6 peaks on TLC system II (J-O by decreasing polarity). Metabolite J co-migrated with E_2 and 17,20αP on TLC and RPTLC. TLC system VI disproved the possibility of J being E_2 or estrone but not 17,20αP. Acetylation provided confirmation of the identity of metabolite J as 17,20αP. Metabolite K was isopolar with 17,20βP on system II and RPTLC. Periodate oxidation yielded a product isopolar with androstenedione and oxidation with 3α20β-HSD yielded a product isopolar with 17P. Metabolites L and N had mobility similar to 17,21P5β on RPTLC but not TLC. 5β-Reduced androstanes were all excluded since they migrate more slowly on RPTLC. On system II, L was less polar than T but more polar than 17P, whereas metabolite N was slightly less polar than 17P. They did not behave as estrogens in system VI (where estrogens have increased mobility in comparison with progestogens and androgens) and seemed not to react with chromium trioxide. Metabolites L and N remained unidentified. Metabolite M was considered to be unreacted precursor since it was isopolar with 17P on RPTLC and TLC, and was not further characterised. Metabolite O had the same mobility of androstenedione on RPTLC and TLC and was partially reduced with sodium borohydride to a compound with the mobility of T but not 3(α,β),17βA5β, 17βA5β, or any of the reduced metabolites of 17P (system III). It was thus identified as androstenedione.

Region RF3 (RF 0.43-0.63) gave nine different peaks on TLC, P-X by decreasing polarity. Metabolites P and R, after oxidation with chromium trioxide, were isopolar with A-3,6,17-trione in system V. After partial reduction with sodium borohydride, P gave one peak and R two peaks, all of them less polar than 6β,17βA on system I. P, R and their reduced derivatives were oxidised with periodate indicating that they were 17α-hydroxylated pregnanes. Also, periodate oxidation of P and of its borohydride reduced metabolite gave products isopolar with 6αA on system II. Periodate oxidation of R and of its less polar borohydride reduced product also gave isopolar compounds but co-migrating with 6βA on system II. The identity of these two androgen products as 6αA and 6βA was

confirmed by acetylation and isopolarity on system V. This was suggestive that P was most probably 6α,17α-dihydroxy-4-pregnane-3,20-dione and R its 6β-epimer. Metabolite Q had an intermediate position with the previous two on TLC system II. Oxidation with chromium trioxide produced a smear on the TLC plate. It remained unidentified. Metabolites S and W were isopolar with adrenosterone on system V after oxidation with chromium trioxide. As metabolite S was isopolar with 11KT on RPTLC and TLC system II, and after acetylation on TLC system III, it was identified as 11KT. Metabolite W was isopolar with 11βA. Metabolite W after reduction with sodium borohydride gave a product isopolar with 11βT and was thus identified as 11βA. Both metabolite T and U reacted with periodate to give products less polar than 6α(β)A or 11βA on system II but more polar than adrenosterone or A-3,6,17-trione. Mobility of T increased moderately after acetylation, suggesting the presence of 1, possibly 2, hydroxyls and U gave 3 products after borohydride reduction suggesting the presence of at least 2 ketonic groups. Together with metabolites V and X, the lack of functional 6-oxygenated standards (especially 5β-reduced) and inconsistent oxidation with chromium trioxide prevented further characterisation of these metabolites.

III.4.3 Metabolite yields

Since only longer incubation periods (18.5 hr) lead to the production of significant amounts of conjugated metabolites, it was assumed that these were most likely artefacts of the static *in vitro* incubation system, which exaggerates the contact between steroids and enzyme systems (see also Ebrahimi *et al.*, 1995) and may not be representative of the *in vivo* situation. Thus, results reported below emphasize the first 4 hr incubation period.

In the free fraction, as with the conjugated fractions, most of the recovered metabolites were 5β-reduced steroids. Of these, the most abundant were 17P5β, 3α,17P5β and 3αA5β. They were produced mainly by the control group, containing mainly testicular tissue, and yield decreased with estrogen dosage (yields for 17P5B, 3α,17P5β and 3αA5β were, respectively, CTL2 - 8±1%, 7.3±0.5% and 4.7±0.6%; T3 - 5.7±1.4%, 6.2±1.1% and

2.4±0.9%; T4 - 2.1±0.7%, 4.3±0.9% and 0.3±0.2%). Separate incubations of testicular and ovarian tissue from group T3 confirmed the testis as the main tissue of synthesis (Fig. 2). In contrast, 3α,17,20αP5β synthesis appeared to be positively correlated to estrogen dosage. Small amounts of 3α,17,20αP5β, its 20β isomer and 3αA5β were always produced during the 1st hour of incubation and increased thereafter. While synthesis of 3α,17,20αP5β was detected at all times during the experiment, the 20β-triol was absent during the 1st week, conspicuous during 6th week and became more abundant at week 14 produced mainly in the testis. The remaining 5β-reduced metabolites had very low yields (<0.5%).

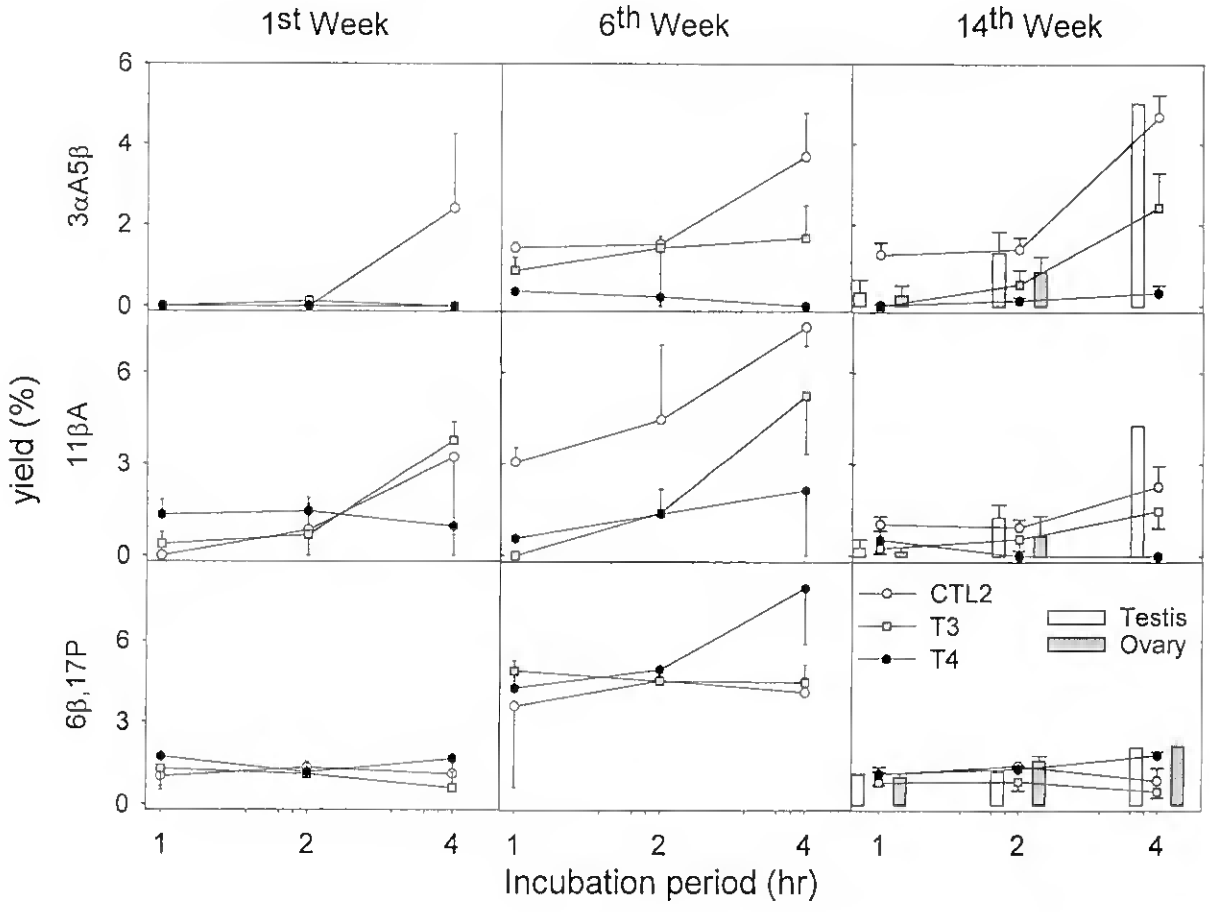

Figure 3. 2- Top and mid panel) Yields of the two major androgens, 3αA5β and 11βA, recovered from incubates of sea bream gonads with [^{3}H]17P as a function of duration of incubation and of estrogen treatment. Lower panel) Yield of 6β,17P, an uncommon fish sex steroid that showed some degree of a positive relationship to estrogen treatment, particularly during the intermediate phases of treatment.

17,20αP and 17,20βP metabolites were absent in the earlier weeks of the experiment but, when present, synthesis was higher in T4 than in the other groups and mainly, but not exclusively, in ovarian tissue. In contrast, 6(α,β)-dihydroxypregnanes were synthesised throughout the experiment. Yields of the 6α isomer (1.5-3.5%) were slightly, but not significantly, higher in T4 than in controls or T3, whereas for the 6β isomer differences were more evident (Fig. 2).

Synthesis of 11βA was clearly inhibited by estrogen treatment. The synthesis of this metabolite was generally more abundant in control gonads, moderate in T3 and much reduced or absent in T4 (Fig.2). Overall, more 11βA was synthesised at week 6, which was coincident with the onset of testicular maturation. The main site of 11βA synthesis was the testis. The synthesis of 11KT followed the same pattern of 11βA but yields were much lower (always less than 2.4%). Androstenedione yields were low and unrelated to treatment with the exception of week 14 when it was high in all groups in the first 2 hr and then decreased sharply indicating further metabolism. There were no differences in androstenedione yields between treatments or tissues.

Unidentified metabolites from RF2 region (L and N) seemed intermediate products in steroid biosynthetic pathway as yields decreased continuously after 1h incubation period. Maximum yields were relatively high (L ~ 3.5% and N ~4.5% at week 14) but unrelated to treatment. Of the remaining unidentified metabolites from RF3 region (Q, T, U, V and X), only yields of metabolite U appeared to increase with estrogen treatment (at week 6, 4h, T4 had: 5.2±0.01%; T3: 3.6±1.2%; CTL2: 3.1±0.2%)

III.4.4 Enzymatic activity

In general, 5β-reductase (5β-Red), 3α-HSD and desmolase were the most active enzymes (Fig. 3). At least, 9 out of the 24 metabolites isolated were 5β-reduced and from these 5 of the more abundant were also 3α-hydroxylated. 5β-reductase and 3α-HSD, which

were equally active, showed increased activity with incubation time in all groups during the first week. In the 6th week they were more active in the control group and by week 14 activity was inversely related to dose of estrogen treatment (Fig.3). The activity was located mainly in the testis as indicated by the separate incubations of the two gonadal tissues of group T3. Desmolase activity was also inversely related to dose of E_2-treatment immediately after the 1st week.

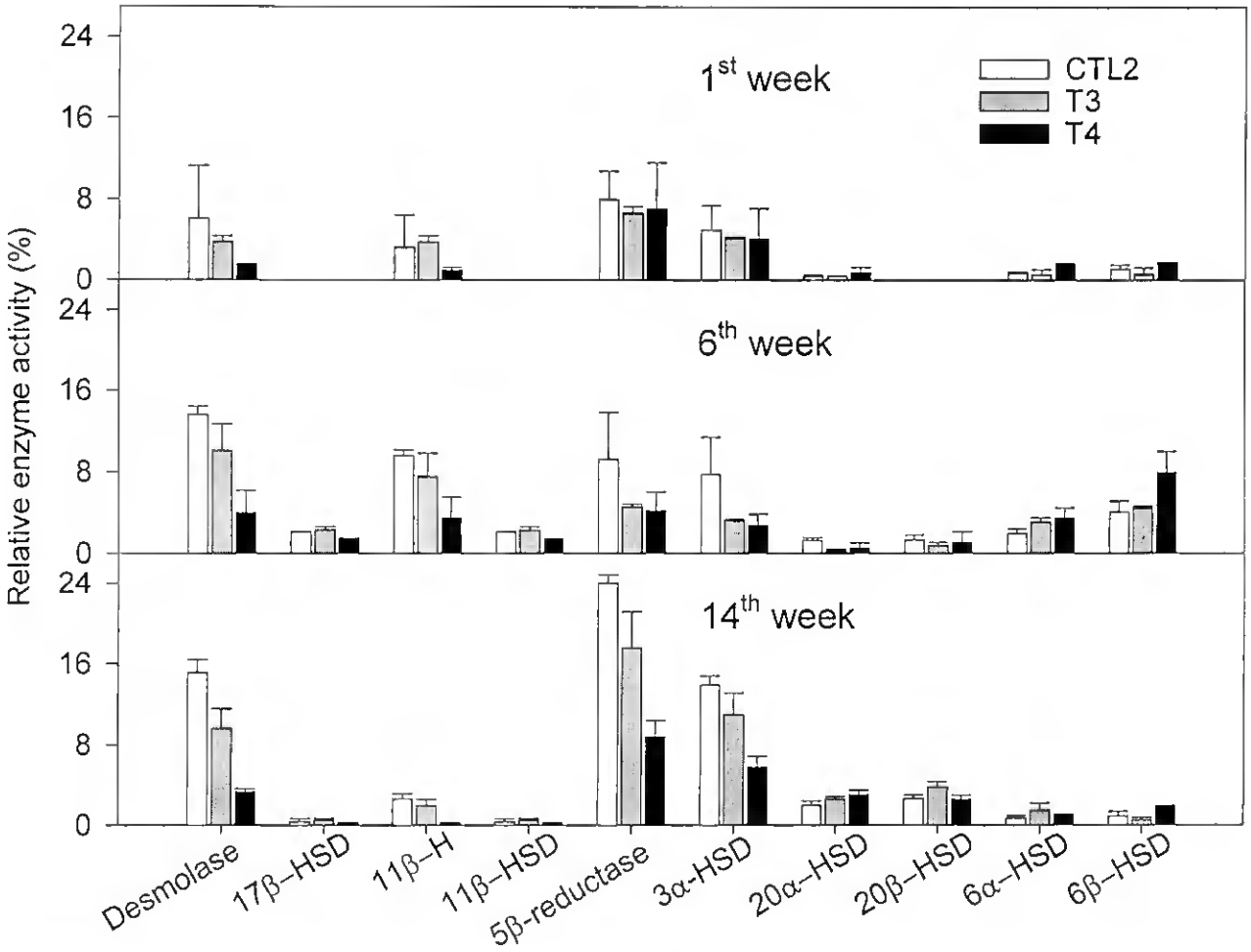

Figure 3.3- Relative activity of steroidogenic enzymes detected after 4 hours incubation of sea bream gonads with [^{3}H]17P. Fish were administered 2 mg.kg-^{1} (T3), 15 mg.kg-^{1} (T4) E_2 and no E_2 (CTL2) in food for up to 14 weeks before the incubations. Relative enzymatic activity was calculated as sum of the percentage yield of steroids metabolised by a particular enzyme.

The same inverse relationship between treatment E_2 dosage and enzymatic activity was found for 11β-hydroxylase (11β-Hyd). However, unlike 5β-reductase and desmolase, highest activity and larger differences between groups were evident at week 6 in CTL2 and T3 (when proliferation of male germ cells beyond the spermatogonia stage occurred) whereas at week 14 (when gonads were ready to emit), it was negligible (Fig. 3). At week 6, a peak in 6β-HSD activity was also noticeable and this was particularly true for the ovarian portion of the gonads (T4).

The activity of the remaining enzymes identified (6α-Hyd, 20β-HSD, 20α-HSD, 11β-HSD and 17β-HSD) was substantially lower, each hardly surpassing 4% of maximum activity.

III.5 Discussion

One of the significant findings of this study was the isolation and identification of a large number of metabolites (17 out of 24 that were identified), and the relationship established between steroidogenic changes and estrogen treatment. The results from the present study are in contrast to previous reports of steroidogenesis in sea bream gonads (Colombo *et al.*, 1972; Eckstein *et al.*, 1978) in which only a few metabolites had been identified, all 4-androsten/pregnen, 5-pregnen and estratrien steroids. The different results obtained between studies are related to the type of precursor used, maturity stage of the gonads, age of the fish and also to the fact that in the early studies only a fraction of the products were actually isolated and analysed (see Colombo *et al.*, 1972). In common with the results reported by Colombo *et al* (1972), in the present study steroidogenesis was greater in testicular tissue than the ovary, as indicated by generally higher recovery of precursor from incubations of mainly (control group) or exclusively testicular tissue (in the incubations of separate tissues from group T3). Similar observations have also been reported in another protandric sparid, *Pagellus acarne* (Reinboth *et al.*, 1986).

The difference in steroidogenic capacity between control and experimental groups was generally quantitative rather than qualitative. The same metabolites were identified in all three groups but estrogen treatment caused a marked reduction in 5β-reduction, 3α-reduction, side-chain cleavage and 11β-hydroxylation (Fig.4), although the latter was less abundant. The change in enzymatic activity paralleled the histological changes observed during estrogen treatment (Condeça and Canario, 1999), which caused a strong inhibition of testicular growth with germ cells not developing beyond the spermatogonia, while ovarian tissue did not appear to be affected. Thus the steroidogenic changes can be ascribed to a general reduction in the steroidogenic capacity of the testis.

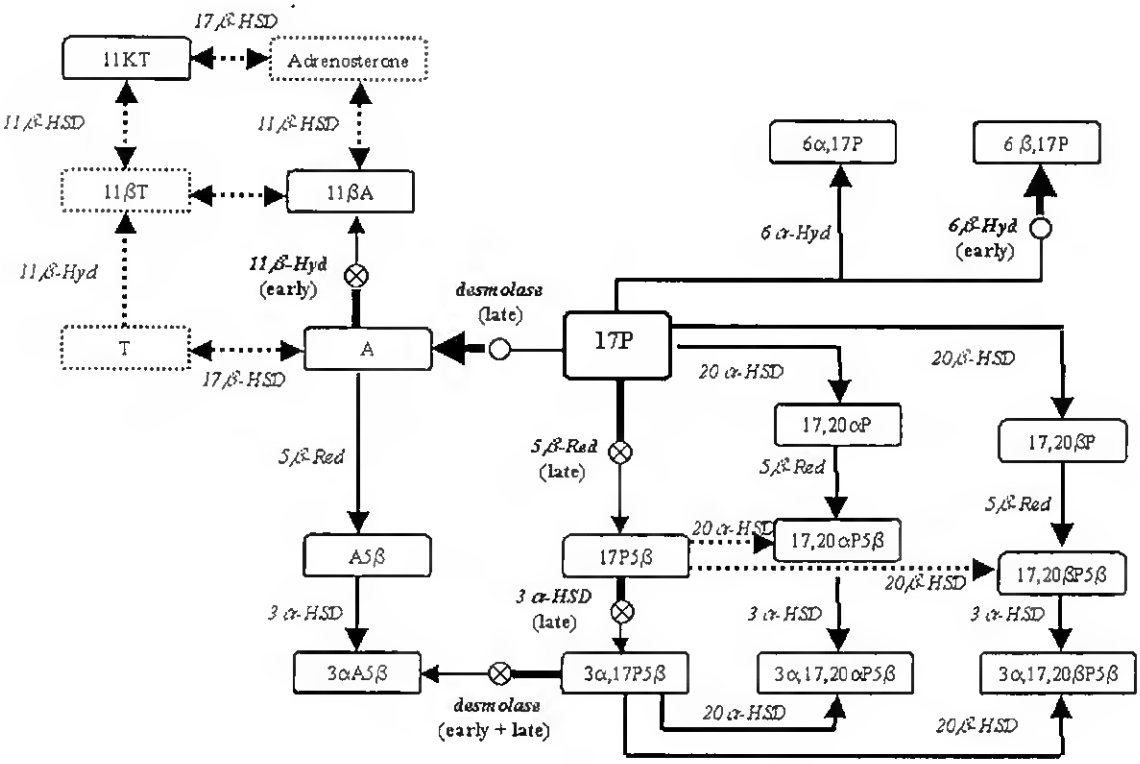

Figure 3.4- Suggested main steroidogenic pathway in sea bream gonads and how it is affected by estrogen treatment. Reduction in enzymatic activity caused by estrogen is marked with ⊗ and enhanced activity is marked with O. In brackets is indicated the timing (early or late in treatment) when changes in enzymatic activity were more pronounced. Dashed arrows and boxes indicate inferred pathways. Legends to steroids is in Table 1. legend to enzymes: *Hyd* – hydroxylase; *Red* – reductase; *HSD*- hydroxysteroid dehydrogenase.

The reduction of 11β-Hyd activity in the group treated with the highest dose of estrogen was simultaneous with the disappearance of spermatocytes in the testis, which is consistent with a role for 11-oxygenated androgens in spermatogenesis in sea bream, as found for other teleosts (Fostier *et al.*, 1983; Borg, 1994; Miura *et al.*, 1996). Of the oxygenated androgens, higher yields were generally obtained for 11βA, a steroid that has been found to be also the major 11-oxigenated male androgen synthesised *in vitro* in several other teleosts, *Gasterosteus aculeatus* (gonochoristic, Borg *et al.*, 1989), *Clarias Gariepinus* (gonochoristic, Schoonen *et al.*, 1987); *Pagellus acarne* (protandric hermaphrodite, Reinboth *et al.*, 1986); *Lates calcarifer* (protandric hermaphrodite, Guiguen *et al.*, 1995); *Epinephelus microdon* (protandric hermaphrodite, Debas *et al.*, 1990). Although in general 11KT is considered to be the most active androgen in teleosts, a stimulatory action of 11βA in the germinal epithelium, on spermatogenesis, on the development of the seminal vesicle and on the expression of secondary sex characters in African catfish has been described (Resink *et al.*, 1987; Schoonen *et al.*, 1987). Evidence from *in vivo* and *in vitro* studies also in *Clarias gariepinus*, showed that 11βA is converted to 11KT in the liver (Cavaco *et al.*, 1997a; Cavaco *et al.*, 1997b), raising the possibility also for some extragonadal control of 11KT production in the sea bream using testicular 11βA as a precursor. Considering that sea bream testis is able to synthesize 11KT (this study and Eckstein *et al.*, 1978) the role of 11βA requires further investigation. In goldfish (*Carassius auratus*) and carp (*Cyprinus carpio*) gills have also been shown to be able of converting 17P to 11KT, possibly to be immediately released in the water in relation to a pheromonal role (Kime and Ebrahimi, 1997). In this context, the testis as a source of precursor for extragonadal transformation of sex steroids for signalling of conspecifics would be a reliable indicator of sexual status, without the danger of exaggerated production of unwanted active steroid.

The synthesis of the androgen 3αA5β was also associated with estrogen treatment with a large production by testicular tissue and negligible production by ovarian tissue. Free and conjugated 3αA5β has been identified in large amounts in incubations of testis gobies, *Gobius jozo* and *Gobius paganellus*, where it was shown to be used to attract females (Colombo *et al.*, 1977; Colombo *et al.*, 1979). In the protandric *Pagellus acarne* it was also

a produced in higher yields by "pure" testis than by "inverting" testis or "pure" ovary (Reinboth *et al.*, 1986). In the present study synthesis of 3αA5β increased with time and was highest at the end of the experiment when larger differences between control and experimental groups were found. Furthermore, most 5β-reduced metabolites gave larger yields at that period suggesting that the importance of 5β-reductase for testicular tissue increased as spermatogenesis progressed. This increased 5β-reduction has been previously associated with testicular tissue in gonochoristic (*Poecilia latipinna*, Kime and Groves, 1986) protogynic (*Coris julis*, Reinboth and Becker, 1984) and protandric (Rhabdosargus sarba, Yeung and Chan, 1985; *Pagellus acarne*, Reinboth *et al.*, 1986) species. Also, incubations of grouper (*Epinephelus coioides)* spermatozoa with 17P as precursor yielded 2-3 fold more 5β-pregnanes than 4-ene metabolites (Lee *et al.*, 1998). It is not clear, however, what would be the physiological role of 5β-reduction, although it as been suggested that it could act as a biological buffer, promoting the synthesis of inactive biological products (Kime and Heyder, 1983) or nonaromatizable products (Quérat *et al.*, 1986).

Of the more polar pregnanes, 3α,17,20αP5β and 3α,17,20βP5β, were detected in significant amounts. The former was present in larger quantities and throughout the experiment. The latter appeared at around the 6th week and correlated mostly with the development of the testis (the main site of its production). These steroids are metabolites of respectively, 17,20βP and 17,20αP, which were also detected in small amounts. While in males 17,20βP is involved in the acquisition of sperm motility and is synthesized by mature spermatozoa (Ueda *et al.*, 1984; Suzuki *et al.*, 1991), it has also been detected in testis without spermatozoa of immature rainbow trout, *Oncorhynchus mykiss* (Vizziano *et al.*, 1995). In females, 17,20βP is produced by the granulosa layer of oocyte follicles and is a mediator of oocyte final maturation (Nagahama, 1997). The production of 17,20βP by sea bream pre-vitellogenic oocytes in the present study is indicative, like in testis, that 20β-HSD protein is expressed at a an early stage. 17,20αP is also produced by sperm of several teleosts including dab, (*Limanda limanda*, Canario and Scott, 1989b), carp *(Cyprinus*

carpio, Asahina et al., 1990), grouper (Epinephelus coioides, Lee *et al.*, 1998) and flounder (*Platichthys flesus*, Asahina *et al.*, 1994) but its function has not been elucidated.

A feature of sea bream gonadal steroidogenesis less common in teleosts was the presence of 6α- and 6β-hydroxylation in the gonads. To our knowledge this has only been described for fathead minnow (*Pimephales promelas*) where 6β-hydroxytestosterone was identified as a minor metabolic product from testosterone with a low clearance rate as compared to other identified steroids (Parks and LeBlanc, 1998) and in female African catfish where 3α,6α,17α,20β-tetrahydroxy-5β-pregnane and 3α,6α,17α,20β-trihydroxy-5β-pregnane-20-one, were identified *in vitro* and *in vivo* and were suggested to have a function in the induction of oocyte maturation and ovulation (Schoonen *et al.*, 1988). In mammalian species, however, 6-hydroxylation is quite common in the final steps of extra hepatic inactivation of 5α-reduced bioactive metabolites of progesterone or testosterone (Chantilis *et al.*, 1996). In our study, only 6β-hydroxylase activity was enhanced by estrogen treatment, although it is not clear whether it has any role during feminisation.

A significant component of water-soluble metabolites of an unknown nature, which did not behave as glucuronides or sulphates, were detected in the incubations. Studies in the protandrous *Lates calcarifer* identified in late transitional gonads a major metabolite (52%) from androstenedione suggestively identified as 3-ester of 17β-estradiol which could only be extracted after hydrolysis with a sodium hydroxide solution (Guiguen *et al.*, 1995). Since in sea bream the proportion of water soluble metabolites of unknown nature increased as the ovarian portion of the gonad increased in relative importance, it would be of interest to determine their nature and whether steroid esters have a role in gonadal feminisation in hermaphrodite teleosts.

In summary, sea bream gonads can synthesize a large range of steroids, some of which have previously been identified in relation to gonadal development and maturation. The key enzymes influenced by estrogen treatment, whose activity may mediate natural sex change are 11β-hydroxylase, desmolase, 5β-reductase and 3α-hydroxysteroid

dehydrogenase (Fig. 4). The mechanism by which steroids bring about modifications in the gonadal tissue that lead to sex change requires further research.

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CHAPTER IV

SEASONAL CYCLE OF GONADAL DEVELOPMENT, STEROIDOGENESIS AND PLASMA SEX STEROIDS DURING THE SECOND YEAR OF LIFE OF SEA BREAM, *Sparus aurata* (L.)

IV Seasonal cycle of gonadal development, steroidogenesis and plasma sex steroids during the second year of life of sea bream, *Sparus aurata* (L.)

IV.1 Abstract

Sea bream in their second year of life and reared at different densities were collected quarterly during two consecutive years from two fish farms in the southern Iberian peninsula (Maresa, 1 fish/m^3 and Aquamarim 3 fish/m^3). Testicular and ovarian cycles were followed simultaneously with gonad steroidogenesis and the plasma profile of several sexual steroids (E_2, T, 11βA, 11βT, 11KT, 17,20αP, 17,20βP, 17,20β,21P and also 5β,3α-reduced metabolites). Gonadal histology showed that the onset of testicular maturation starts in August and that the sperm duct becomes full of spermatozoa in November. From January to April, the testis exhibits signs of regression including degenerative foci in which germ cells were replaced by tissue with yellow-brown appearance. The ovary becomes the predominant tissue during this period although it does not grow significantly.

No correlation between sexual steroids and morphological or cytological parameters were found, possibly because of the influence environmental factors such as rearing density on the endocrine pattern. The major plasma androgen found was 11βA, which, in fish from Aquamarim, increased in concentration from August to September with a peak in the spermiation period suggesting a possible role in this process. Results from *in vitro* steroidogenesis and radioimmunoassay showed a strong 5β-reductase activity in sea bream gonads. A striking difference in ir17,20αP profile between fish farms was observed but its significance remained unclear. A combination of RIA and chromatography showed that the immunoactive metabolite(s) was very polar.

KEY WORDS: sex reversal, gonads, 11β-hydroxyandrostenedione, 5β-reduced steroids

Running title: Seasonal cycles in sea bream second year of life

IV. 2 Introduction

Unlike eutherian mammals, where gonadal sex is highly stable and unaffected by sex steroids (Nagai, 1992), fish gonadal development is highly plastic (Shapiro, 1992). The diversity of patterns of sexual development include fish that differentiate at the earliest juvenile stages as their final sex (gonochorists), simultaneous hermaphrodites in both juvenile and adult phases, and sequential hermaphrodites, with gonads differentiating and functioning first as ovaries and latter redifferentiating as functional testes (i.e. protogynous) or vice-versa (i.e. protandrous) (Shapiro and Rasotto, 1993). Gonadal sex reversal is, thus, a common phenomenon in teleosts. The morphological changes associated with sex reversal have been well described for several species, including the sea bream *Sparus aurata* (Zohar *et al.*, 1978), but information is lacking about physiological parameters such as plasma sexual steroid levels and/or gonadal steroidogenesis. Studies of sex inversion have generally compared only male and female stages reflecting more their respective reproductive characteristics than the regulation of sex inversion itself (Baroiller *et al.*, 1999). Furthermore, ready availability of E_2, T and 11KT antisera and the generalised importance of these steroids for oogenesis and spermatogenesis (Nagahama, 1994) has meant that emphasis has been given to them rather than other sexual steroids of possible relevance for the sex inversion process. Clearly integrative studies of histology, gonadal steroidogenesis and blood plasma profile of sex steroids are of great interest in studying fish undergoing sexual inversion.

Sexual steroid involvement in protandric sex inversion has been studied in several sparids. In *Rhabdosargus sarba* seasonal plasma profiles for E_2, estrone, androstenedione, T, 11KT and 11βT were observed for fish at different sexual phases (Yeung and Chan, 1987). Although differences were found in plasma sexual steroid content it was suggested that they reflected mainly the reproductive activity in the female and the male rather than a causative hormonal factor controlling sex reversal. However, previous *in vitro* data showed that transitional gonads had a high 5β-reductase activity (Yeung and Chan, 1985). In the tropical seabass *Lates calcarifer* the ratio of plasma androgens/estrogens was higher in males than in transitional fish which had strikingly high E_2 levels (Guiguen *et al.*, 1993;

Guiguen *et al.*, 1995). *In vitro* data, however, showed that transitional gonads did not synthesise E_2 from androstenedione but rather an estrogen-like metabolite. The authors suggested that this shifted metabolism could be an estrogen reservoir mechanism caused by high endogenous E_2 (Guiguen *et al.*, 1995). Also, both *in vitro* and *in vivo* data showed that in this species there was testicular specific 11-oxoandrogen synthesis suggesting that sex inversion could be associated with a shift in gonadal steroidogenesis from 11-oxygenated androgens to estrogens. In two other protandric fish, similar schemes have been proposed to explain sex reversal. In the black porgy *Acanthopagrus schlegeli* both 11KT and E_2 (together with T) had seasonal peaks during the spawning season, respectively in males and females. It was suggested that in reversing fish 11KT synthesis was shifted to T, which can be metabolised to E_2 through aromatase activity (Chang *et al.*, 1997). Estrogen was suggested to exhibit positive feedback to stimulate GnRH and GtH expression and release, further increasing aromatase activity. In the sobaity, *Sparidentex hasta*, seasonal serum concentrations of 11KT, 11βT and E_2 correlated well with the change of sexual status of the gonads during regression and recrudescence suggesting that E_2 may be involved in sex inversion also in this species (Kime *et al.*, 1991). Furthermore, the 11-oxygenated androgens assayed were considered reliable indicators of the occurrence of sex reversal but the author emphasised the need of preliminary studies with radiolabelled precursors to determinate if other steroids are of quantitative or qualitative importance in that process.

The objective of the current study was to detail gonadal morphological changes during sea bream natural sex reversal and to correlate these with the plasma sexual endocrine profile and the steroidogenic capacities of the gonad. Such data has not previously been reported for 2-year old sea bream with the exception of E_2. In fish with gonads with a predominant testis, E_2 was highest during the early spermatogenic phases (745±142 pg ml^{-1}) but low in males with running milt (155±11 pg ml^{-1}) whereas in predominantly ovarian gonads, E_2 was highest in the early vitellogenic phase (1669±142 pg ml^{-1}) and low in the resting phase (108±11 pg ml^{-1}) (Kadmon *et al.*, 1985). Older ovulatory females, however, contained large plasma quantities of 20β-reduced pregnanes and also of 17,20β,21P, suggested as the oocyte maturation inducing steroid for the sea bream (Canario *et al.*, 1995). A detailed study with radiolabelled 17P on sea bream gonadal steroidogenesis

during estrogen induced sex reversal revealed new insights on putative important endogenous steroids (see chapter III). We suggested that sex reversal is characterized by the "switching-off" of some important enzymes that appear to dominate steroidogenesis of the male gonad. Basically, during initial stages of sex reversal there was a substantial decrease in 11βA synthesis and in the later stages 5β3α-reduced steroids, especially 3αA5β, synthesis was also reduced. Other 20α/β reduced were also produced by sea bream gonads. In the present study in addition to E_2 and T, 11-oxygenated androgens (11βA, 11βT and 11KT) and 20 α/β reduced pregnanes (17,20αP, 17,20βP and 17,20β,21P) were also monitored. Moreover, antisera produced to detect steroids with "5β3α" and "5β21" configuration were also used to assess if 5β-reduced steroids detected *in vitro* were produced *in vivo*.

IV.3 Materials and Methods

Fish - Twelve sea bream were caught at each sampling event from outdoor ponds of two different fish farms using a hand operated net. The fish-farm stations chosen were Maresa, Mariscos de Esteros S.A. (Ayamonte-Spain) and Aquamarim, Aquacultura de Marim, Lda (Olhão-Portugal). Rearing densities were 1 and 3 fish/m^3, respectively. Sampling was made quarterly from June 1993 to November 1995. All fish were aged 16 to 20 months and selected to weigh as close as possible to maximum commercial weight (300g).

Sampling procedure - Fish were anaesthetised with 0.25 ml/L of 2-phenoxyethanol and fork length (nearest mm) and weight (nearest g) were taken. Blood samples were collected from the caudal vein with heparinized syringes, centrifuged at 12000 rpm for 5 minutes, and the plasma was frozen in liquid nitrogen and kept at -20°C until analysis. Fish were then killed by decapitation and the gonads were removed and weighted (nearest mg). A fraction of the gonad was fixed in Bouin's solution for 36 hours for histology and another fraction (ca. 15mg) was taken for *in vitro* steroidogenesis.

Histology and image analysis - Tissues were embedded in paraffin wax, sectioned transversally (6μm) and stained with Erlich's hematoxylin and eosin (H & E). Transverse sections were taken from the posterior quarter to middle region of the gonad. A preliminary study showed that the proportions of both testicular and ovarian tissues are statistically similar for the different parts of the gonad (Table 4.1). Image analysis was carried out (OPTIMAS 5.2, Bioscan, Incorporated – USA) to measure testicular and ovarian areas within sections and their cellular component areas, e.g., oogonia, oocytes, spermatogonia, etc. Undifferentiated connective tissue, central cavities, main blood vessels and fat tissue were only considered for gonad total area. Cellular component measurements were made by random sampling of three $6x10^4$ μm^2 areas. The diameters of the 10 largest pre-vitellogenic oocytes present in the cross section were also measured.

Table 4.1 – Testicle and ovary relative areas in gonad cross-sections from anterior to posterior zone in male (fish 1-3), ambisexual (fish 4-6) and female (fish 7-9). One way repeated measures analyses of variance (after inverse sine transformation of percentages) indicates that there is not a statistically significant difference in the proportion of either testicular or ovarian tissue in relation to the position in the gonad.

	Testis area (%)			**Ovary area (%)**		
Fish	anterior	middle	posterior	anterior	middle	posterior
1	92.80	79.30	91.30	6.50	7.50	6.20
2	81.10	96.40	87.60	15.90	3.60	9.40
3	85.40	95.20	95.20	13.30	15.50	4.30
4	70.20	82.20	89.15	25.70	10.70	9.60
5	67.30	75.70	89.20	30.40	20.70	10.30
6	94.60	92.00	85.60	2.80	5.90	13.30
7	0.80	0.30	2.40	91.70	96.40	94.70
8	0.20	0.20	2.20	98.70	97.80	95.00
9	0.80	0.40	0.30	90.50	98.40	96.70
	p=0.532			**p=0.189**		

The gonadosomatic (GSI) index was calculated according to the formula:

$$GSI\,(\%) = \frac{\text{gonad weight (g)}}{\text{fish total weight (g)}} \times 100$$

Testicular and ovarian indexes were calculated as the product of GSI by the proportion of testicular or ovarian tissue in cross-sections.

Steroid assays - Free, glucuronide and sulphated fractions from blood plasma (100 µl) for radioimmunoassay were obtained using methodologies described by Scott and Canario (1992) and Canario and Scott (1989). Specificity tables for all RIAs used are given in Table 4.2. The limit of detection of the assay was 400 pg/ml for the 17,20αP, 17,20βP and 17,20β21P assays, 200pg/ml for T and 11KT assays, and 50pg/ml for 11βT, 11βA and E_2 assays (for steroid abbreviations refer to list in page 55 and Table 3.1). Intra-assay and inter-assay precision (coefficient of variation) were, respectively, 5.0% and 12.0% for 17,20αP, 4.6% and 21.5% for 17,20βP, 7.2% and 16.9% for 17,20β,21P, 7.5% and 12.4% for T, 8.2% and 11.6% for 11KT, 13.0% and 9.5% for 11βT, 7.8% and 17.5% for 11βA and 6.9% and 8.3% for E_2. Two other RIAs, using antisera previously reported by Canario & Scott (1990), one detecting progestogens with a 5β-reduced, 3α-hydroxylated configuration ("5β,3α") and another detecting 5β-reduced, 21-hydroxylated configurations ("5β,21") were also used (Inbaraj *et al.*, 1997; Scott *et al.*, 1997). Label and standard steroid used for "5β,3α" RIA was 3α,17,20βP5β and for "5β,21" RIA were, respectively, 3α,17P5β or 3α,17,21P5β.

In vitro steroidogenesis - Transverse fractions (10-20 mg) of gonads were incubated *in vitro* in Costar 12 well plates (Costar Corporation, Cambridge, USA) containing 1ml trout balanced salt solution (Jalabert and Fostier, 1984), 5 I.U. human chorionic gonadotrophin (hCG) to stimulate steroidogenesis and either androstenedione or 17α-hydroxyprogesterone as precursors (0.5 $\mu g.ml^{-1}$). No cofactors were used. Incubations were carried out for 48h at room temperature (21 ± 2°C) in a humid, oxygenated

atmosphere and under constant gentle agitation. Incubates were stored at -20°C for later extraction and GC-MS identification.

Derivatization and GC-MS identification – Incubates from fish belonging to the same sampling date were pooled together and then extracted with diethyl ether for free, sulphated and glucuronide fractions using the same methodology as for blood plasma analysis. These conjugate fractions were also pooled since the objective of the study was to obtain a general picture of seasonal effects on gonad steroidogenic capacity. TMS and OTMS derivatives were prepared by mixing the metabolites with methoxyamine hydrochloride (2.5mg) in pyridine (50µl) and allowing to react for 1h at 100°C. Pyridine was removed by evaporation under N_2 and trymethylsilylimidazole (100µl) was added. The sylilation was conducted at 100°C for 1h. Steroid derivatives were analysed with a Varian GC-MS (GC: Star 3400cx; MS: Saturn3; Varian chromatography systems, U.S.A.) with a 15-meter DB5ms non-polar fused silica capillary column. Injection mode and metabolite identification were carried out as indicated in chapter III. Mass spectral data were acquired by scanning the 55-650 mass range. Steroid yields were estimated by the integral calculus of the area under the metabolite peak in the GC chromatogram and then expressed as percentage of total steroid area.

Statistics – Data are presented as mean ± SEM. Seasonal data obtained over two years were pooled and presented as an annual cycle. Differences in oocyte diameter were tested with non-parametric Kruskal-Wallis analysis of Variance followed by Dunn's all pairwise comparisons. Plasma steroid content was tested with one-way analysis of Variance (ANOVA) followed by Tuckey's Honestly Significant Difference test. Plasma steroid contents were also correlated with other variables from histology and morphometry (e.g. gonadosomatic index or estimated spermatozoon weight, *etc*) in a matrix of Pearson product-moment correlation coefficients with adjusted Bonferroni probabilities. Statistical significance was considered at the 5% level.

IV.4 Results

IV.4.1 Seasonal alterations of the gonads

Gonadosomatic, testicular and ovarian indexes – The evolution of the gonadosomatic index was parallel in the two fish farms. Sea bream from fish farm Maresa always had highest GSIs. Low values (<0.2%) predominated in the non-breeding season, from January to September (Fig.4.1). During this period, the ovarian portion of the gonad tended to predominate (maximums of 75±3% and 58±4%, respectively in Maresa and Aquamarim, in April; Figs. 4.4a and 4.4b). However, the ovarian index did not reflect this predominance since, although the index grew significantly as compared to November, the increase was less than 0.1% of body weight as the whole gonad had shrunk after the breeding season (Fig. 4.3). Both testicular and ovarian indexes ranged between 0.01-0.1% during this period except for a small increase in the ovarian index at Maresa to nearly 0.15%.

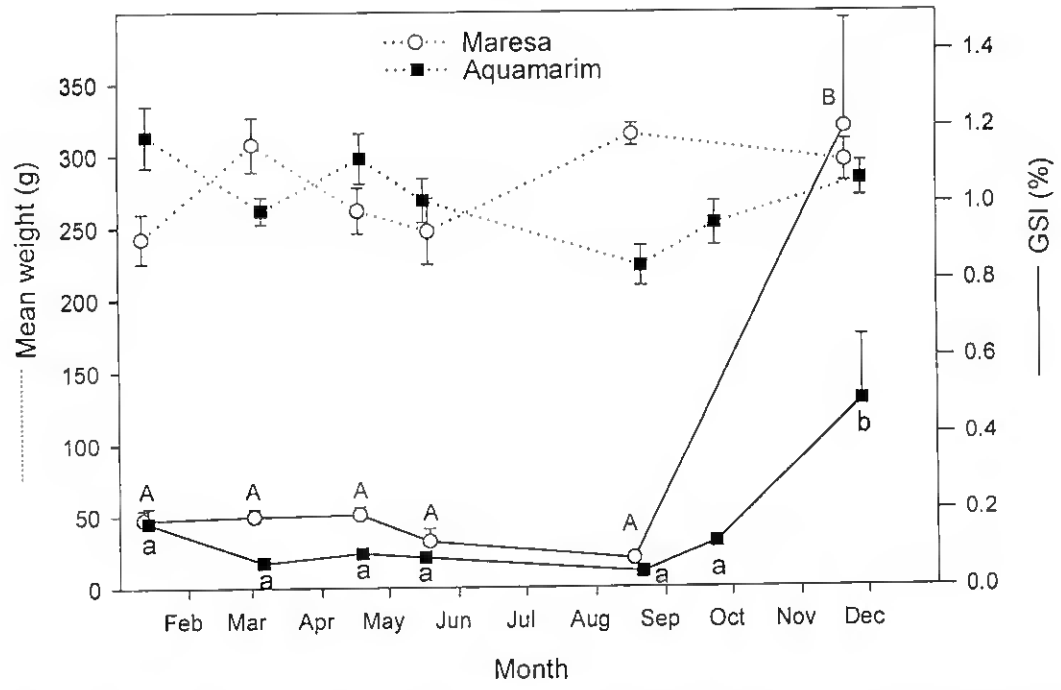

Figure 4.1 – Seasonal changes in fish mean weight and gonadosomatic index at both fish farms. Different capital letters above circles and small letters below squares indicate statistically seasonal differences in GSI ($p<0.05$), respectively for Maresa and Aquamarim. Note the gap in the GSI value in December (spawning season) between fish farms.

Large GSI increases occurred during the breeding season (October to December). Temperature presumably did not influence GSIs differences between the two fish farms since, during the whole sampling period, similar maximal and minimal water temperature values were observed in both farms (Fig.4.2). Concomitantly with the larger testicular portion of the gonad during the breeding season (> 85% of cross-sections area), large testicular indexes revealed the prominence of the testis over the ovary (Fig. 4.3).

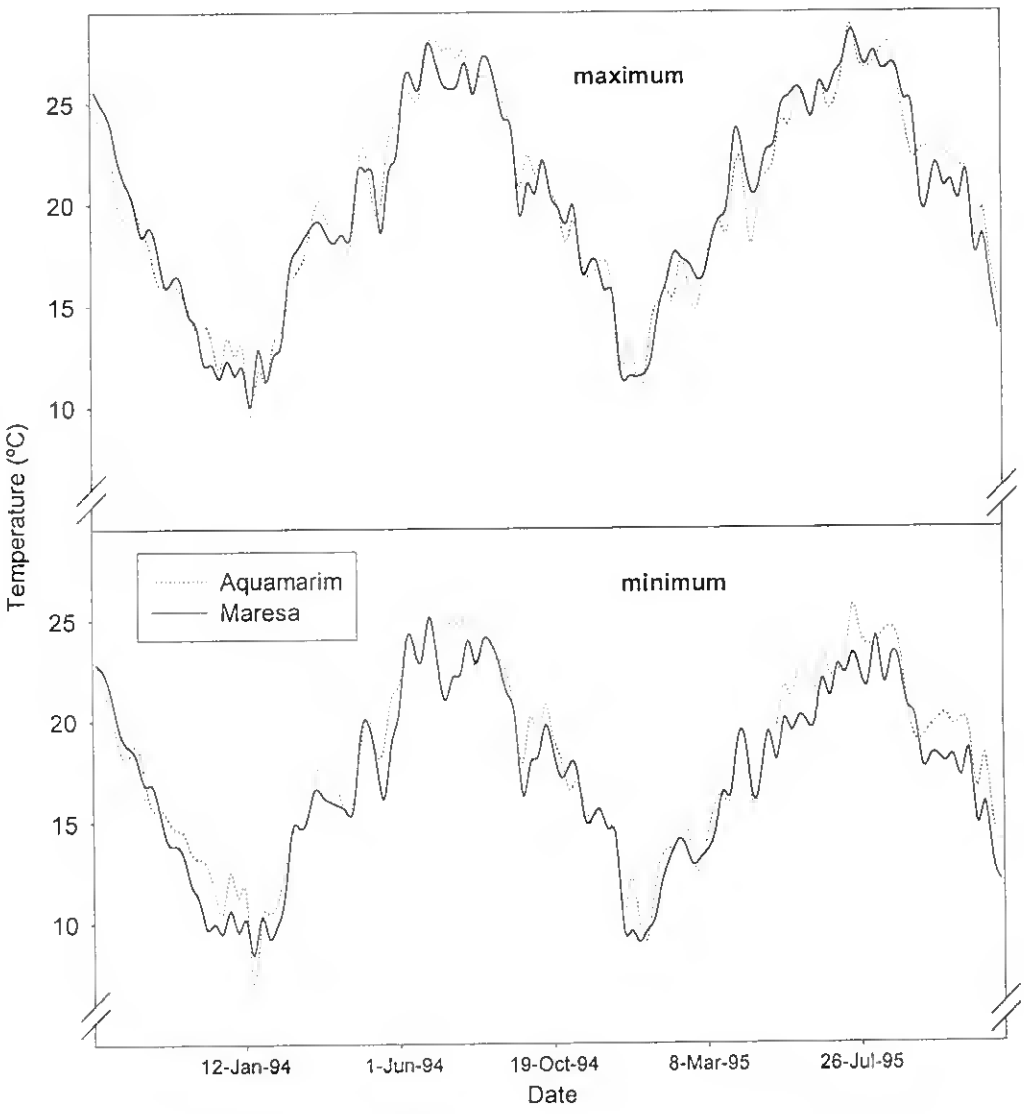

Figure 4.2 – Maximal (upper panel) and minimal (lower panel) water temperatures in Maresa and Aquamarim fish ponds during experimental period.

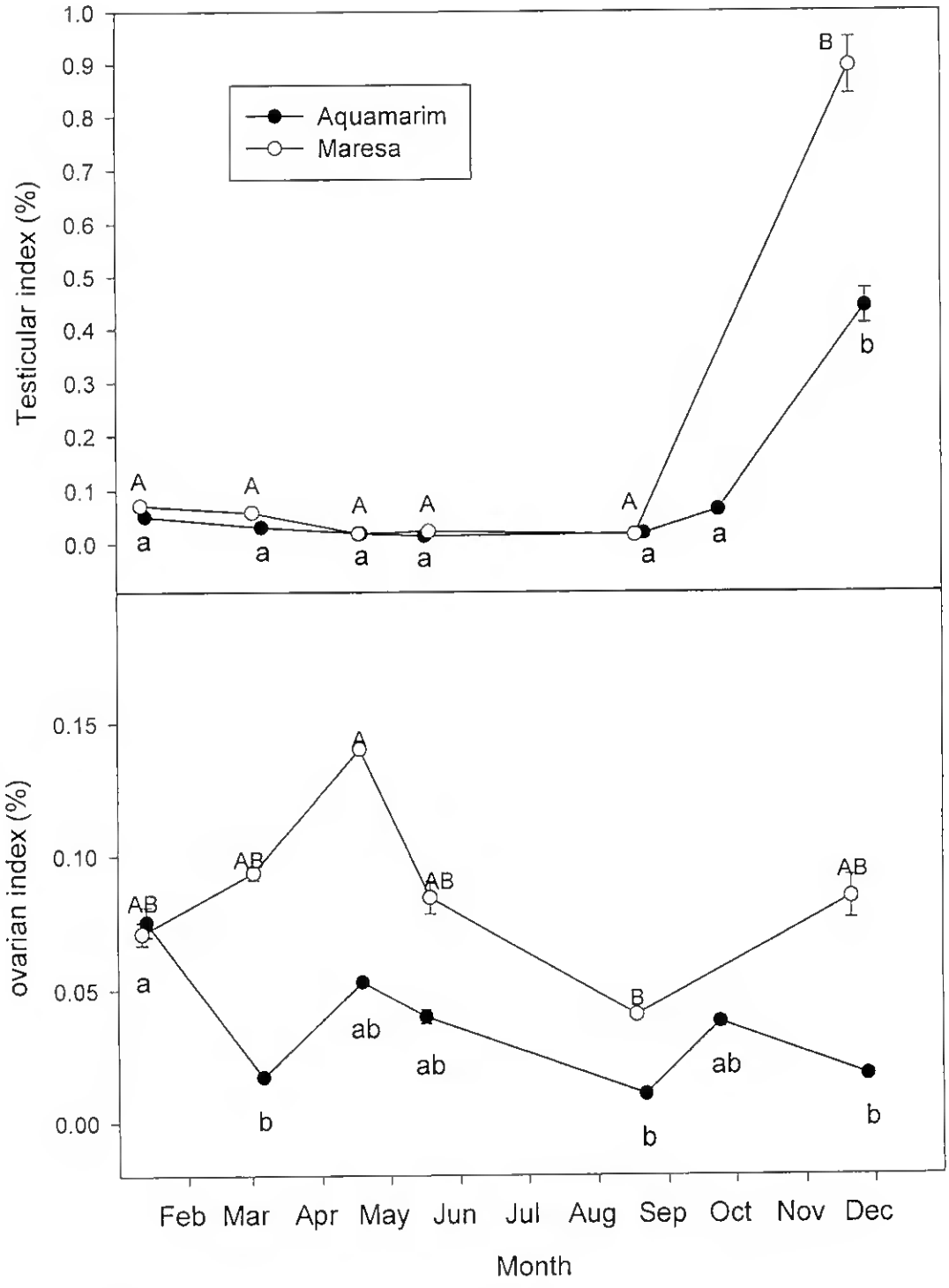

Figure 4.3 - Progression of testicular (upper panel) and ovarian (lower panel) indices. The two indices were calculated by multiplying the gonadosomatic index by the proportion of each type of tissue determined from histological sections. Different capital letters above circles and small letters below squares indicate statistically seasonal differences ($p<0.05$), respectively for Maresa and Aquamarim. Note difference in scale.

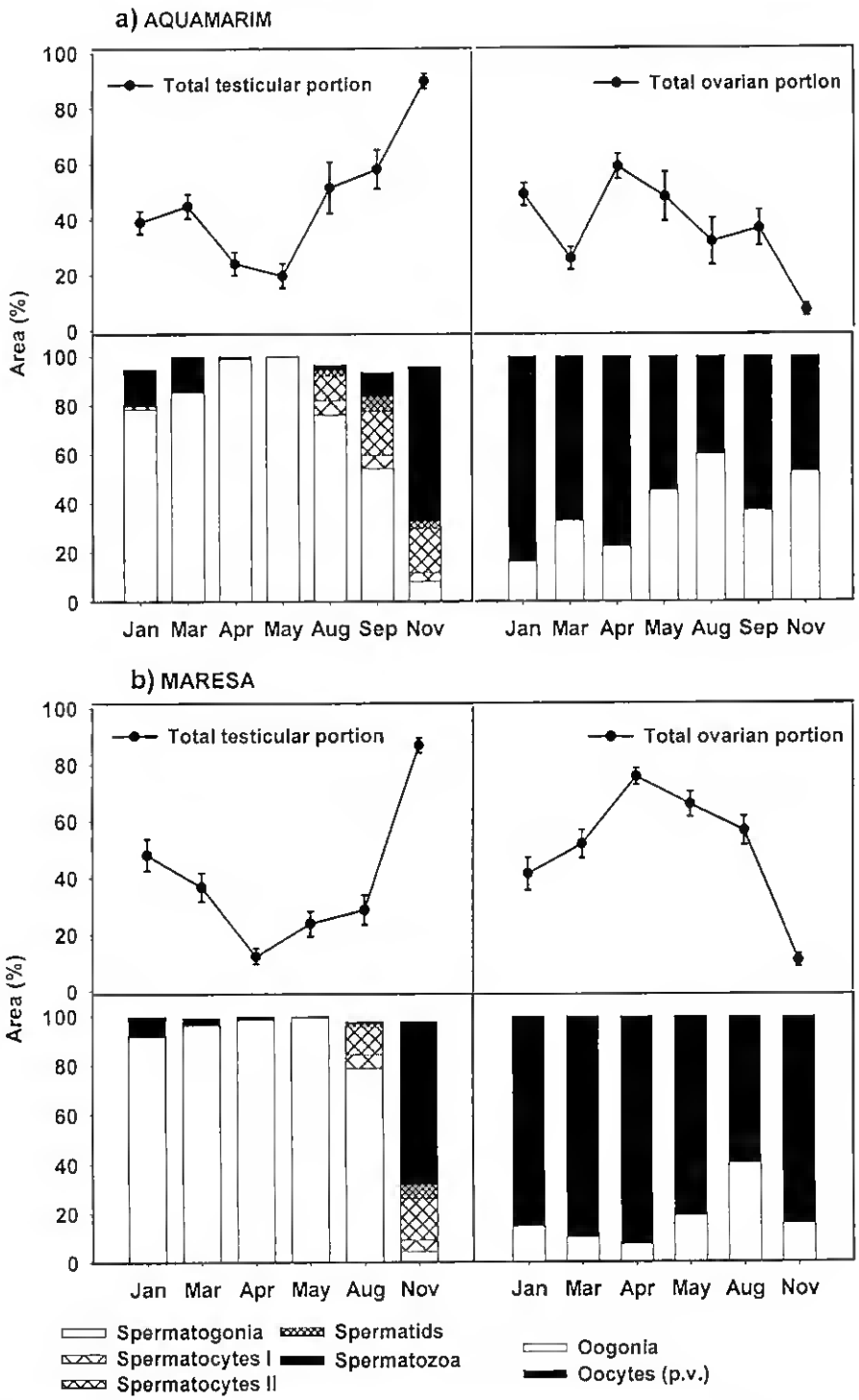

Figure 4.4 - Seasonal variations of testicular and ovarian relative areas of the gonad cross-sections (upper panel), and correspondent cytological content (lower panel) in fish from Aquamarim (a) and Maresa (b). In general, testicular organization was similar in both fish farms reflecting one period of increase of relative size and cellular diversity prior to the spawning season (November) followed by a regression period where spermatocytes are no longer recruited (January-April). In contrast, the ovary showed an increasing relative size during testis regression but the oogonia/oocyte content was not so uniform.

Testis histology- Testicular organisation in terms of cellular diversity and quantity was similar in fish from both fish farms. In August, cellular diversity increased as spermatocytes (I and II) and spermatids became more abundant. Spermatozoa were also present but still vestigial. By September, spermatozoa increased to occupy nearly 10% of the cross sectional area of the gonad (Fig. 4.4a and 4.5a) and the sperm duct was formed. In November the testis were fully mature and ready to emit, with the sperm duct full of spermatozoa (>60% area), and all fish released sperm when a slight pressure was put on the abdomen (Fig. 4.5b). In contrast, spermatogonia were at their minimum (<10%; figs. 4.4a and 4.4b). In January-March, the area of spermatozoa decreased to nearly 10% with spermatozoon cysts becoming partially empty. Spermatocytes and spermatids were no longer being recruited indicating a post-emission situation. The first signs of testicular disorganisation appeared and were indicated by a shorter and more irregular outline of the seminiferous lobules and the presence of an increasing number of connective tissue cells (probably immune such as leucocytes, fibroblasts, etc) and fibres in the middle of the testis. In April, testicular disorganisation was at its maximum as connective cells spread all over the testis, seminiferous lobules became shorter and more detached from the testicular wall (Fig. 4.5c). During this period the testis were reduced to their smaller relative size and the ovary reached its maximum (figs. 4.4a and 4.4b). In addition, several degenerative foci where germ cells where replaced by connective tissue with yellow-brown ("yellowish") appearance became visible (Fig. 4.5d). In May, the testis reassumed features of organisation since degenerative foci as well as the connective cells disappeared and seminiferous lobules regained their longer configuration and proximity of the testicular wall.

Ovary histology- The development of the ovary in fish from the two fish farms was more heterogenous than the testis. The largest difference was observed in the numbers of oogonia and ovarian index from fish caught in March at Aquamarim (figs. 4.4a and 4.4b). In general, the relative area of oogonia increased from April to August-November and decreased in the post-spawning period (January-April), whereas pre-vitellogenic oocytes showed the opposite pattern. The changes in the diameter of the largest oocytes (Fig.4.6) nearly followed the pre-vitellogenic oocyte area, i.e., biggest oocytes are present in the ovary from January/March to April/May.

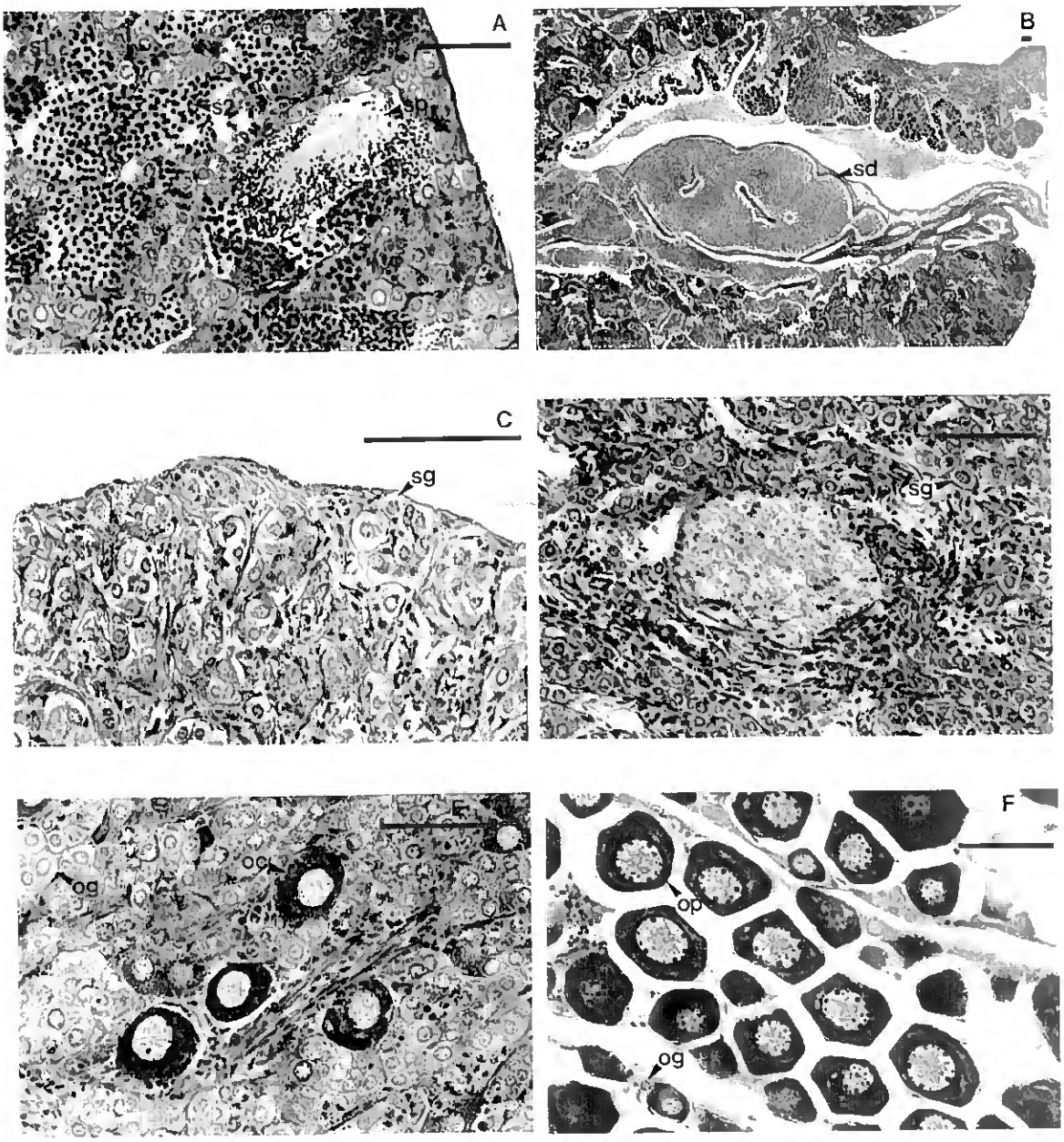

Figure 4.5 – Major seasonal effects in gonadal histology of fish from both fish farms (stained with H & E). In September the sperm duct was already formed and overall spermatozoan content was approx. 10% (A). In November, the testes were fully mature and ready to emit as the sperm duct (sd) was filled with sperm (B). Testis regression began in January with spermatozoon cysts (sp) becoming partially empty, reaching maximum disorganization in April when seminiferous lobules became shorter and irregularly outlined (C) and large degenerative foci of "yellow-brown" appearance were observed (D). In the ovary, smaller pre-vitellogenic oocytes in the chromatin-nucleolar stage (oc; E) were more abundant during the spawning period than perinucleolar oocytes, which predominated from March to April (op; F). Bar in photo corresponds to 50 μm. Legend: spermatogonia (sg); spermatocytes I and II (s1, s2); oogonia (og).

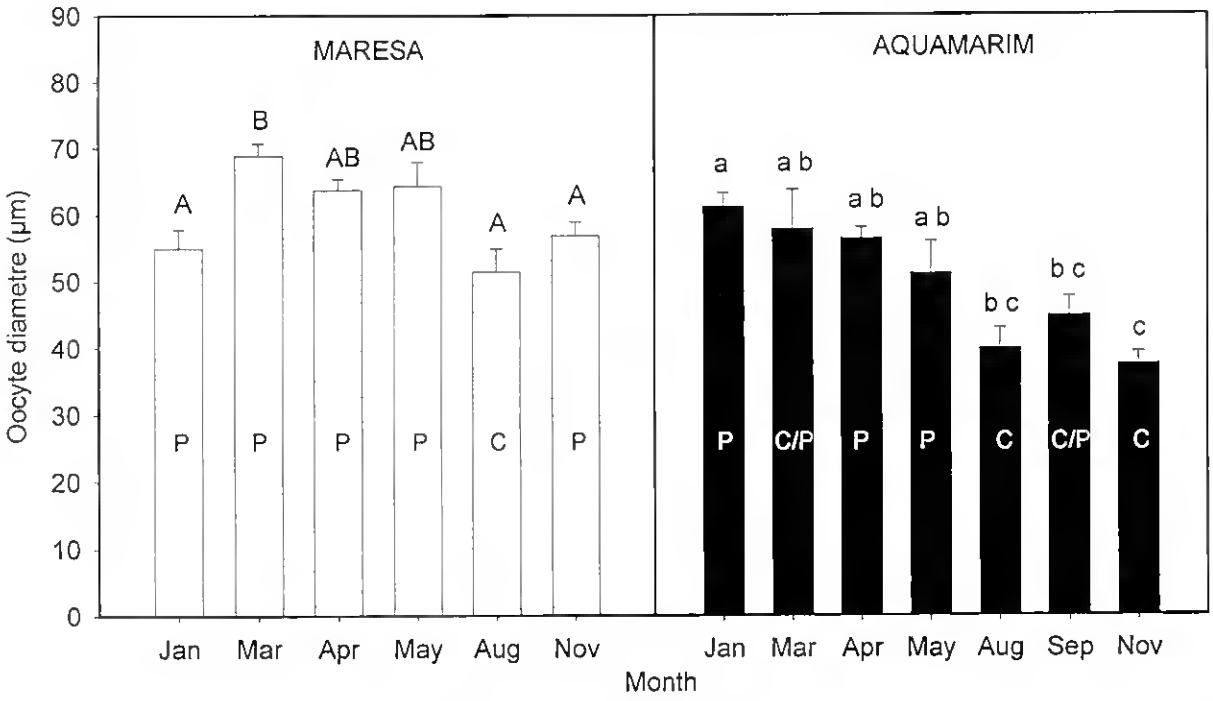

Figure 4.6 – Seasonal variation of the mean diameter of the 10 largest pre-vitellogenic oocytes in cross-sections. Letters C and P within bars indicate, respectively, predominant chromatin-nucleolar or perinucleolar stages in oocyte development. Different letters above bars indicate statistically different diameters ($p<0.05$; non parametric Kruskal-Wallis analyses of variance) within Maresa (capital letters) or Aquamarim (small letters). Note that, in general, oocytes tended to be bigger and in a more advanced stage of development after spawning.

These larger oocytes were at a more advanced stage (perinucleolar; Fig. 4.5f), than most of the smaller ones (chromatin-nucleolar stage; Fig. 4.5e), which predominated in August-November (spawning period), indicating oocyte growth after spawning.

IV.4.2 Sexual Steroids

Specificity of antisera - Cross-reactivities of the antisera used in this study, either reported before, or assayed in the present report, are shown in Table 4.2.

Table 4.2- Cross-reactivity of steroids with the antisera used in this study. Cross-reactions are expressed as percentage at the 50% level of displacement as compared to the standard steroid unless otherwise stated. * indicates the percentage of displacement at 100pg level.

	Radioimmunoassay									
Systematic name	"5β,3α"	"5β,21"	17,20βP	1720β21P	17,20αP	T	11βT	11KT	11βA	E_2
5β-pregnan-3,20-dione					<0.003					
3α-hydroxy-5β-pregnan-20-one	8.9	10.7								
17α-hydroxy-5β-pregnane-3,20-dione	0.2	42.8								
20α-hydroxy-5β-pregnan-3-one					0.12 (30%) 0.14 (50%) 0.7 (70%)					
20β-hydroxy-5β-pregnan-3-one					<0.015					
3α,17α-dihydroxy-5β-pregnan-20-one	96.1	73.1								
17α,21-dihydroxy-5β-pregnan-3,20-dione	0.2	24.1			<0.007					
3α,17α,21-trihydroxy-5β-pregnan-20-one	64.1	standard								
3α,17α,20α-trihydroxy-5β-pregnane	44.5	4.1			0.5 (30%) 2.6 (50%) 6.5 (70%)					
3α,17α,20β-trihydroxy-5β-pregnane	standard	1.4	<0.006	0.06						
3β,17α,21-trihydroxy-5β-pregnan-20-one	0.1	0.4								
3α,6α,17α-trihydroxy-5β-pregnane-20-one	0.2									
3α,17α,20β,21-tetrahydroxy-5β-pregnane	100	<0.02	<0.006	0.2	<0.002					
3α,17α,20β,21-tetrahydroxy-5β-pregnane-11-one	3.4	<0.02								
3α,11β,17α,20α-tetrahydroxy-5β-pregnane	25				<0.006					
3α,11β,17α,20β-tetrahydroxy-5β-pregnane	25			<0.005	<0.002					
3α,17α,20α,21-tetrahydroxy-5β-pregnane					<0.015					
3α,11β,17α,21-tetrahydroxy-5β-pregnane-20-one	15.9	<0.02								
3α,6α,17α,20α-tetrahydroxy-5β-pregnane	0.2				0.03					
3α,6α,17α,20β-tetrahydroxy-5β-pregnane	1			<0.008						
3α-hydroxy-5β-androstan-17-one	8.7	<0.02								
3α,17β-dihydroxy-5β-androstane	3.5	0.5								
3α,6α-dihydroxy-5β-androstan-17-one	<0.03					<0.01		<0.005		
17α-hydroxy-5α-pregnane-3,20-dione	<0.02	<0.2								
3β,17α,21-trihydroxy-5α-pregnan-20-one	<0.02	<0.02								
3α,17α,20β-trihydroxy-5α-pregnane			3	0.2						
3α,17α,20α-trihydroxy-5α-pregnane					2.6 (30%) 4.7 (50%)					
3β,17α,20α-trihydroxy-5α-pregnane					5.1 (30%) 7.4 (50%)					
3β,17α,20β-trihydroxy-5α-pregnane			54.9	3.5						
3α,17α,21-trihydroxy-5α-pregnan-20-one	1.6	<0.02								
3β,17α,20β,21-tetrahydroxy-5α-pregnane			0.85	81.8						
17β-hydroxy-5α-androstan-3-one						31				
3α-hydroxy-5α-androstan-17-one						0.04				
4-pregnene-3,20-dione			<0.003	<0.001	<0.008	<0.01				<0.2
17α-hydroxy-4-pregnene-3,20-dione			0.025	0.12	<0.015	<0.01				
20α-hydroxy-4-pregnene-3,20-dione			<0.006	<0.03						
20β-hydroxy-4-pregnene-3,20-dione			0.13	0.03		<0.01				
21-hydroxy-4-pregnene-3,20-dione						<0.01				
17α,20α-dihydroxy-4-pregnen-3-one			1.2	0.2	standard					
17α,20β-dihydroxy-4-pregnen-3-one	<0.02	<0.02	standard	4	0.12	<0.01				
17α,21-dihydroxy-4-pregnene-3,20-dione	<0.02	<0.02	<0.006	0.32	<0.015	<0.01				
17α,21-dihydroxy-4-pregnene-3,11,20-trione							4.4*	1.3*		
17α,20β,21-trihydroxy-4-pregnen-3-one			0.4	standard	<0.005					
17α,20α,21-trihydroxy-4-pregnen-3,11-dione					<0.02					
17α,20β,21-trihydroxy-4-pregnen-3,11-dione			0.12	0.6						
17α,20α,21-trihydroxy-4-pregnen-3-one			<0.006	0.03	0.9 (30%) 1.2 (50%)					
6α,17α,21-trihydroxy-4-pregnen-3,20-dione				<0.02						
6β,17α,21-trihydroxy-4-pregnen-3,20-dione				<0.03						
11β,17α,21-trihydroxy-4-pregnen-3,20-dione	<0.02	<0.02	0.05	0.001		<0.01	3.6*	3.3*		<0.2
11β,17α,20β,21-tetrahydroxy-4-pregnen-3-one			<0.05	0.6						
11β,17α,20α,21-tetrahydroxy-4-pregnen-3-one					<0.007					
4-androstene-3,17-dione						0.4			3.16	<0.2
4-androstene-3,11,17-trione							31.8*	76.9*	4.12	
4-androstene-3,6,17-trione						<0.1		<0.004		
17β-hydroxy-4-androstene-3-one			<0.001	<0.003	<0.004	standard	1.4	1.3	0.36	<0.2
17β-hydroxy-4-androstene-3,11-dione	<0.02	<0.02				2.4	4.6	standard	0.39	
11β-hydroxy-4-androstene-3,17-dione							41.4*	30.1*	standard	
6β-hydroxy-4-androstene-3,17-dione						<0.04		<0.001		
11β,17β-dihydroxy-4-androsten-3-one						8.9	standard	2.2	3.95	
6α,17β-dihydroxy-4-androsten-3-one						<0.1		<0.06		
6β,17β-dihydroxy-4-androsten-3-one						3.1 (30%) 8.4 (50%)		<0.07		
17β-hydroxy-1,4-androstadiene-3-one							52.1*	52*		
3β-hydroxy-5-pregnene-20-one										<0.2
3β,17α-dihydroxy-5-pregnen-20-one	<0.02	0.01	<0.006	0.2						
3β,17α,20β-trihydroxy-5-pregnene			50	1.30						

	Radioimmunoassay									
Systematic name	"5β,3α"	"5β,21"	17,20βP	1720β21P	17,20αP	T	11βT	11KT	11βA	E_2
3β-hydroxy-5-androstene-17-one ?						0.05				<0.2
3,17β-dihydroxy-1,3,5(10)-estren			0.002			0.05				standard
3-hydroxy-1,3,5(10)-estrene-17-one						0.01				15
3,17β-dihydroxy-1,3,5(10)-estren-16-one										8
3,16α,17β-trihydroxy-1,3,5(10)-estren-3-one										0.7
3,16α-dihydroxy-1,3,5(10)-estrene-17-one										<0.2

Note: some cross-reaction results are taken from previous reports (11βT and 11KT: Kime and Manning, 1982; T: Scott *et al.*, 1984; 11βA: Schulz, 1985; 17,20βP and 17,20β21P: Canario *et al.*, 1989; "5β3α" and "5β21": Inbaraj *et al.*, 1997).

Since 17,20αP antiserum had not been characterized before and owing to the high immunoactivity in blood of Aquamarim fish (Fig.4.10), RPTLC was carried out on pooled plasma samples (n=10) from fish caught in August and November at Aquamarim. Fractions of 0.5cm of the RPTLC lane were eluted and assayed. In August, more than 90% of the immunoactivity did not coincide with the 17,20αP position, indicating cross-reaction with another steroid related to 17,20αP (Fig. 4.7). The same pooled samples when tested with the "5β3α" antiserum showed high immunoactivity in the same region in August, and also in November, but, unlike with the17,20αP antiserum, the other RPTLC fractions also registered considerable immunoactivity. The overall immunoactivity of the "5β21" antiserum was, however, low. Nevertheless, two small peaks were detected in August overlapping 17,20αP immunoactivity (fig 4.7). The high immunoactivity in the 7-8 cm region of the RPTLC was, most likely, due to more than one steroid because, 1) that activity was detected with three different antisera (Fig. 4.7), and 2) the "5β3α" steroids were present in the two periods whereas "17,20αP" steroid(s) was present only in August. Moreover, when these high activity fractions were rerun on normal phase TLC, with and without previous treatment with the enzyme 20β-HSD, there were no differences in the "5β3α" chromatograms which showed two main peaks of immunoactivity more polar than 3α,17,20αP5β. These results suggest that this region contain at least 2 very polar steroids with 3 or 4 hydroxyl groups. It is likely that one of those hydroxyl groups has 20α conformation because of their cross-reaction with the "1720αP" antiserum and because the enzyme 20β-HSD did not modify these steroids. The possible position of the other hydroxyl groups of these compounds could be 6α or 11β since, from our experience with RPTLC, steroid mobility is to a large extent associated with the positions of the hydroxyl

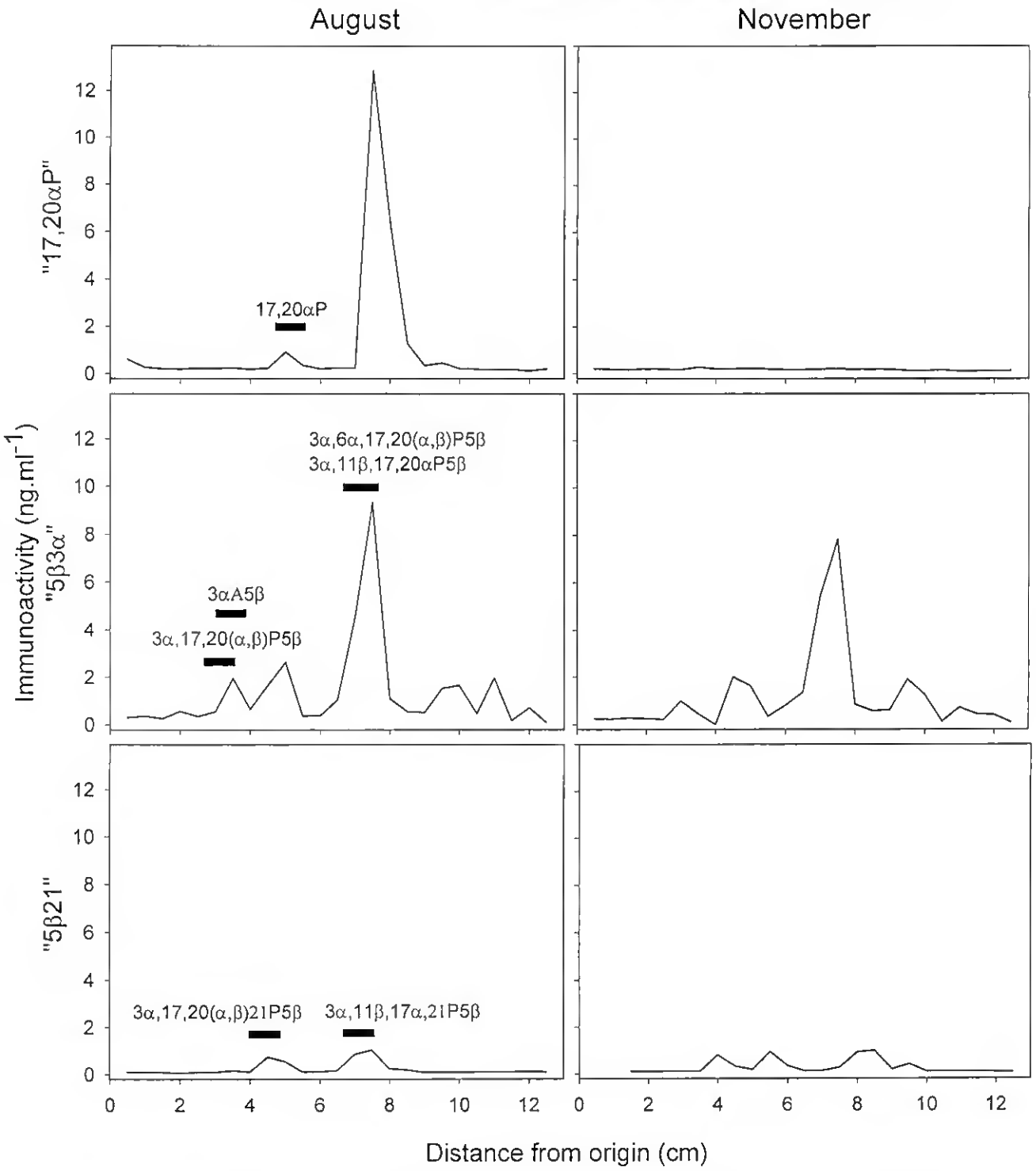

Figure 4.7 – Immunoactivity of pooled plasma samples collected in August and November from Aquamarim fish. Plasma was fractionated by RPTLC and fractions assayed by RIA with "17,20αP", "5β3α" and "5β21" antisera. Bars over chromatogram indicate approximate elution position of standards. Note the high immunoactivity of fractions in the 7-8cm region with all antisera suggesting the presence of several very polar steroids possibly including 20α, 11β and/or 6α hydroxyls.

(and ketonic) groups. For example, steroids such as 3α,6α,17α,20αP5β, and in particular 3α,11β,17α,20αP5β or 3α,11β,17α,21P5β have similar chromatographic properties as the unidentified steroids and cross-reacted to some extent with the "5β3α" RIA (respectively, 0.2%, 25% and 16%; see Table 4.2). Also, they were all identified from *in vitro* metabolism of 17P (Table 4.3). However, many other possibilities, including steroids with both 6α and 11β hydroxyls, were unavailable and could not be tested.

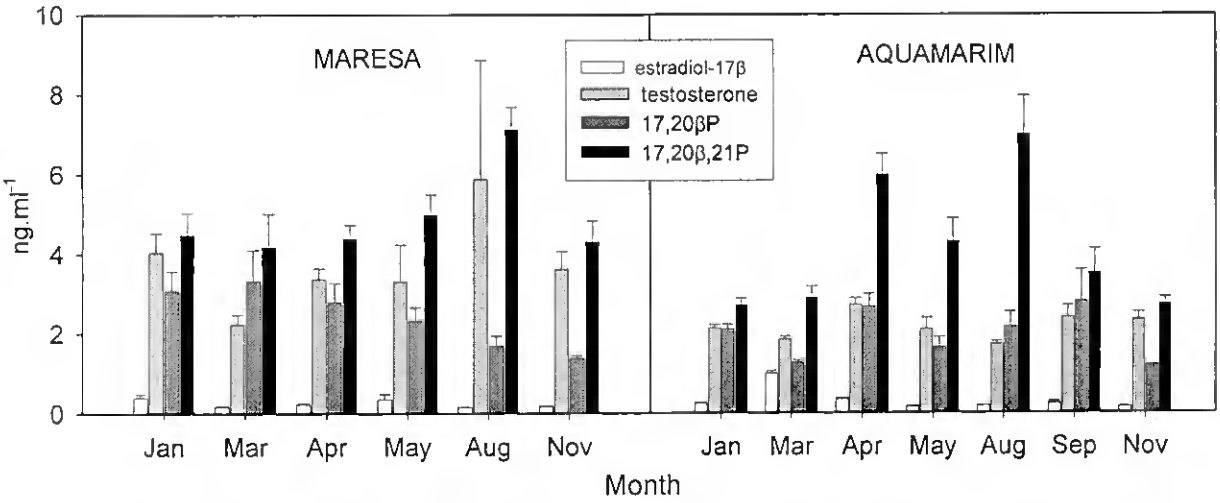

Figure 4.8 – Seasonal levels of plasma E_2, T, 1720βP and 1720β21P in fish collected from Maresa and Aquamarim. E_2 was significantly higher ($p<0.05$) in January, at Maresa, and in March, at Aquamarim, Testosterone showed no significant seasonal differences but was present at higher mean concentrations than estrogen. 17,20βP levels were significantly higher during the post-spawning (January-April) than in the spawning period (November) except in March at Aquamarim. The plasma level of 17,20β,21P was statistically higher at the onset of testicular maturation (August) than the rest of the year (except in May at Maresa and May/April at Aquamarim).

Plasma steroids - The majority of steroids assayed had levels near the low detection limit (0.15 $ng.ml^{-1}$ for E_2, 0.6 $ng.ml^{-1}$ for androgens and 1.2 $ng.ml^{-1}$ for progestogens,) or their annual profiles was apparently unrelated to season. For example, E_2, 11βT and 11KT levels (Figs.4.8 and 4.9) were always very low in both fish farms and did not change significantly except in January (at Maresa) and in March (at Aquamarim) when a peak of E_2 was detected ($p<0.05$). Testosterone and 17,20βP levels were higher than estrogen (Fig.

4.8) but no significant seasonal changes of T were observed, whereas 17,20βP was higher during the post-spawning season (January-April) as compared to the spawning period (November; $p<0.05$). The major androgen (up to 8ng.ml^{-1}) in blood plasma was 11βA but seasonal profiles were different (Fig. 4.9) between fish farms. Fish from Maresa tended to have higher levels of 11βA than fish from Aquamarim. Levels of 11βA were observed to increase during the pre-spawning and spawning periods (August-November) and peaked in March when they were significantly higher than at other times of the year. Fish from Aquamarim had a peak in April and in September, both significantly different from May and November values.

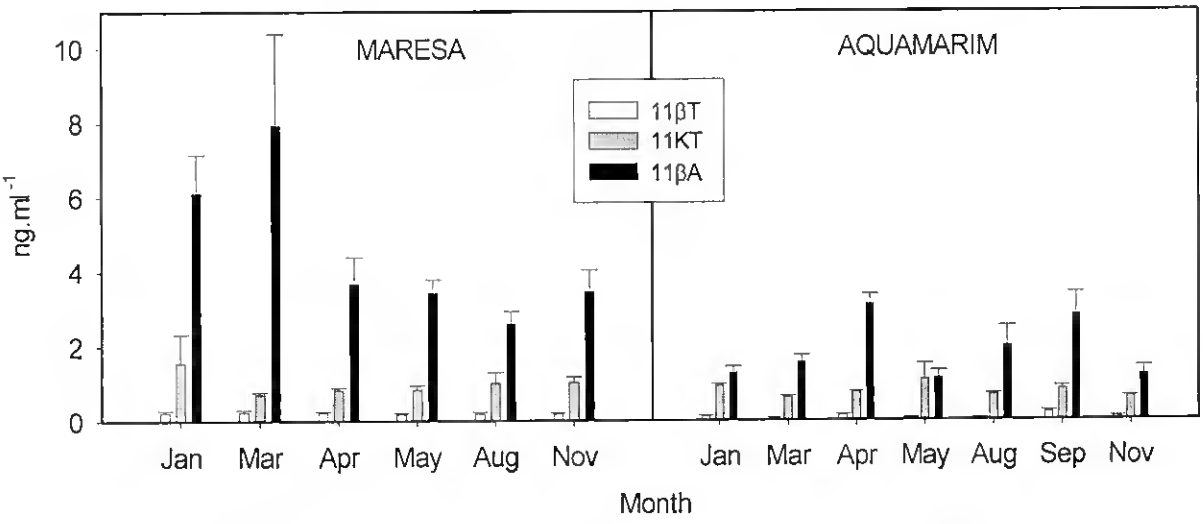

Figure 4.9 - Seasonal levels of plasma 11-oxygenated androgens at Maresa and Aquamarim. 11βT had levels at the detection limit whereas 11KT levels were higher but did not change. 11βA was the major 11-oxoandrogen with seasonal profiles different between fish farms. At Maresa, March was significantly higher ($p<0.05$) than August and November (January was also higher than November) but at Aquamarim both April and September peaks were statistically higher than May and November (April was also higher than January and March levels).

Seasonal profiles of 17,20β21P in fish from both farms were similar with a maximum at the onset of testicular maturation (August; 7.1±0.6 and 7.0±1.0 ng.ml^{-1} in Maresa and Aquamarim, respectively) significantly higher than levels of most of the year ($p<0.05$; except May in Maresa and May/April in Aquamarim). The most striking observation was,

however, the very different profile of ir17,20αP in the two fish farms. Maresa levels were always at the detection limit while at Aquamarim they reached more than 15 ng.ml^{-1} during May (free + conjugate). Also, unlike the remaining hormones assayed, free steroid was practically absent whereas conjugate levels was mainly sulphated (Fig.4.10). The seasonal 17,20αP profile at Aquamarim showed decreasing levels from May to November and an isolated peak in March. Total levels of ir17,20αP (free+conjugate) in March, May and August were statistically higher ($p<0.05$) from the rest of the year.

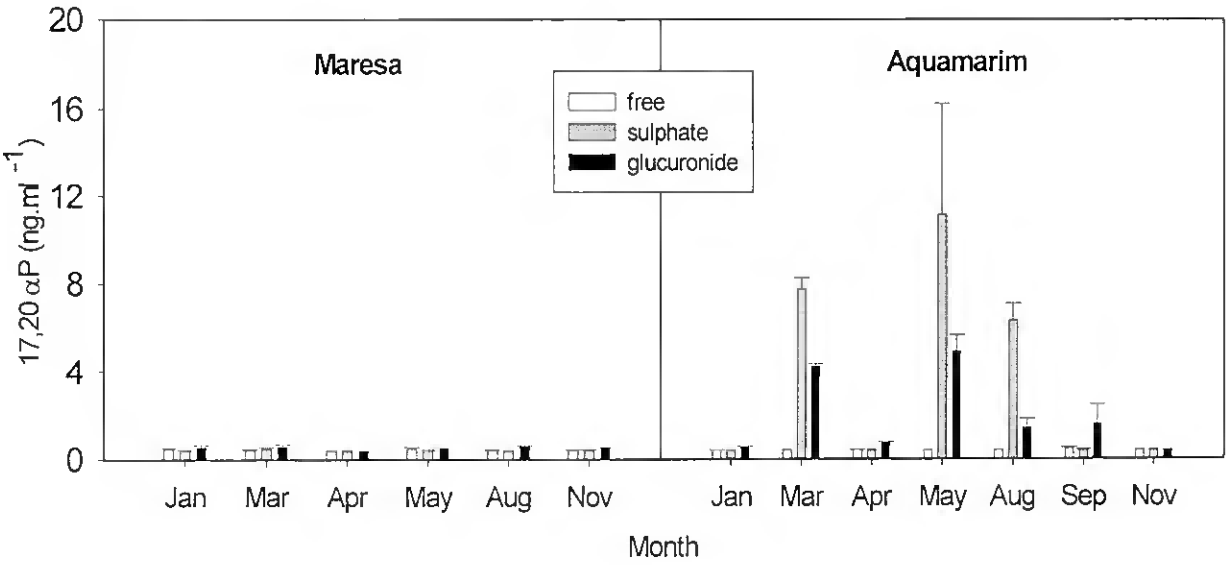

Figure 4.10 - Seasonal plasma levels of free and conjugated forms of immunoreactive 17,20α at Maresa and Aquamarim Practically no hormone was detected in plasma from fish from Maresa, in contrast high conjugate levels were detected in fish from Aquamarim. Total levels (free+conjugate) in March, May and August were statistically different ($p<0.05$) from the rest of the year.

No relationship was found between plasma steroid levels and histological observations. Only the correlations between 11βT and 11βA ($r>0.480$; $p<0.05$) or 17,20βP and 17,20β21P ($r=0.823$ in Aquamarim and $r=0.531$ in Maresa; $p< 0.01$) were significant in both farms.

In vitro steroidogenesis - When 17P was used as the precursor, 3α,17,20αP5β was the major steroid synthesised (60-90% of total metabolites) by the gonads of fish from both farms during the whole year. An exception was observed with the gonads collected from Maresa in November, where the quantity of this metabolite dropped dramatically from 85 % to less then 35%. Simultaneously, there was a peak in 3α,17,21P5β (13.5%) and of its 11β-hydroxylated form (30.4%) whereas at Aquamarim the maximum yield for these steroids was also in November but always below 6%. An unidentified metabolite X (Fig. 4.11 and Table 4.3) with a pronounced 255 mass peak characteristic of 5(β,α)-pregnanes with hydroxyl groups at C3, C17, C20 (with or without C21 hydroxyl) was also synthesised. Over 10% yield of this metabolite coincided with the period of testicular post-emission or regression while peaks of 20-30% yield coincided with a large ovarian development (April-May). During testicular recrudescence the synthesis of this metabolite was negligible.

The most interesting feature when androstenedione was used as the precursor was the profile of 11-oxoandrogens. 11βA yields increased after the post-spawning period (January) until the onset of testicular recrudescence (May-August) when male diversity of germ cells was at its maximum. These peaks reached over 50% yield and had a sudden drop during spawning (November). The low 11βA yield coincided with a rise in two other 5β-reduced 11-oxoandrogens: 3α,17βA5β-11one and 3α,11βA5β-17one (Fig. 4.11). The yield of the corresponding 11-deoxygenated androgens followed a parallel pattern (3α,17βA5β and 3αA5β; Table 4.3). In common with the progestogen precursor, an unidentified metabolite (Y) with a prominent characteristic ion at 393 was preferentially synthesised during testicular regression (Table.4.3) but no inference could be made about its molecular structure.

Enzymatic activity was mainly attributed to 5β-reductase (5β-Red) and 3α-hydroxysteroid-dehydrogenase (3α-HSD) when 17P was used as a precursor (70-100%) with preponderance during the August-November period. Desmolase activity was low (always<6%) in all the gonads studied but 11β-hydroxylase (11β-Hyd) and 21- hydroxylase

Table 4.3 - Yields and MS parameters of 17P and androstenedione gonadal metabolites identified by GC-MS. Steroid yields were estimated by the integral calculus of the area under the metabolite peak in the GC chromatogram and then expressed as percentage of total steroid area.

Steroids from 17P gonadal metabolism												
						Yield (%)						
Steroid (abbrev.)	Fit	R.Fit	Pur.	R.time (min)	Farm	Jan	Mar	Apr	May	Aug	Sep	Nov
3α,17βA5β	990	862	862	16.619	Mar.	0	0	0	0	0	-	3.2
					Aqua.	0	0	0	0	0	0	0
3αA5β	993	833	833	17.076	Mar.	0	0	0	0	0	-	0.7
					Aqua.	0	0	0	0	0	0	0
3α,17αA5β-11one	998	828	828	20.384	Mar.	0	0	0	0	2.0	-	1.0
					Aqua.	0	4.3	0	0	0.6	0	0
3α,17P5β	997	798	798	21.687	Mar.	0	3.8	2.2	6.0	2.8	-	6.4
					Aqua.	3.5	2.1	4.0	4.4	8.1	6.3	7.4
3α,11βA5β-17one	971	753	747	22.099	Mar.	0	0	0	0	0	-	0.8
					Aqua.	0	0	0.8	0	0.3	0	0
3α,17,20βP5β	979	859	850	23.025	Mar.	0	5.6	3.3	3.0	2.1	-	1.3
					Aqua.	1.9	2.8	0.0	0	1.4	2.7	3.4
3α,17,20αP5β	996	874	874	23.831	Mar.	87.8	71.6	70.6	58.0	84.8	-	32.0
					Aqua.	72.6	58.1	69.5	89.1	77.5	80.9	79.9
3α,17,21P5β	994	763	763	26.058	Mar.	0	0	1.3	0	0	-	13.6
					Aqua.	1.5	1.4	0.7	0.0	0.6	0	3.6
3α,6α,17α,20αP5β	902	723	678	26.507	Mar.	0	0	0	0	0	-	0
					Aqua.	1.1	1.5	0	0	0.2	0	0
11βA	994	688	688	26.545	Mar.	0	0	0	0	0	-	0
					Aqua.	0	0	0	0	0.4	0	0
3α,11β,17α,20αP5β	921	803	748	27.221	Mar.	0	0.0	1.8	0	0	-	0
					Aqua.	1.1	1.7	1.1	1.9	0.3	0	0
3α,17,20αP5β-11one	958	757	739	27.442	Mar.	0	0	1.0	0	0	-	0
					Aqua.	1.3	4.9	1.0	0	0.7	0	0
X	-	-	-	27.79	Mar.	12.2	13.5	8.8	28.8	1.8	-	7.8
					Aqua.	14.2	7.2	23.0	3.0	1.3	10.1	0
3α,11β,17α,21P5β	979	628	623	30.25	Mar.	0	0	2.1	0	0	-	30.4
					Aqua.	0	0	0	0	1.8	0	5.7
11β,17P-3,20dione	990	735	735	32.914	Mar.	0	0	0	0	0.0	-	0
					Aqua.	0	0	0	0	2.1	0	0
Non-identified (Sum-X)	-	-	-	-	Mar.	0	5.5	8.9	4.2	6.5	-	2.6
					Aqua.	2.9	15.9	0	1.5	4.7	0	0
Steroids from Androstenedione gonadal metabolism												
3α,17βA5β	990	862	862	16.619	Mar.	33.4	8.9	10.5	9.7	0	-	21.9
					Aqua.	18.5	0	12.9	0	6.3	5.3	15.7
3αA5β	993	833	833	17.076	Mar.	15.2	8.4	14.4	9.3	6.4	-	17.6
					Aqua.	24.8	6.9	11.6	0	3.5	9.4	13.4
Y	-	-	-	17.964	Mar.	4.3	25.2	9.2	16.3	10.3	-	7.4
					Aqua.	16.0	0	12.9	31.1	0	7.7	0
3α,17αA5β-11one	998	828	828	20.384	Mar.	39.1	19.1	24.1	17.3	16.7	-	22.6
					Aqua.	19.6	56.4	29.0	0	13.4	48.8	22.3
3α,11βA5β-17one	971	753	747	22.099	Mar.	5.4	6.1	11.3	5.2	7.3	-	15.3
					Aqua.	11.2	17.8	14.1	0	12.8	21.6	18.8
11βA	994	688	688	26.545	Mar.	0	15.2	20.2	27.7	52.5	-	6.7
					Aqua.	3.8	14.5	11.5	63.0	58.5	5.7	13.9
Non-identified (Sum-Y)					Mar.	2.6	17.2	10.4	14.6	6.8	-	8.4
					Aqua.	6.0	4.4	8.0	5.9	5.3	1.4	16.0

(21-Hyd), whose activities never surpassed 10% yield, achieved a significant expression in November in fish from Maresa (32 and 44%, respectively). When androstenedione was used as the precursor, 5β-Red and 3α-HSD were again found to be important enzymes since their yields varied from 30 to 90% but, in contrast to the situation observed with 17P as substrate, 11β-Hyd (35 to 90%) and 17β-HSD (15 to 70%) were also very active. In general, 11β-Hyd reached its maximum activity during testicular recrudescence, May-August/September, which contrasted with what was observed for 5β-Red/3α-HSD and 17β-HSD activities whose yields dropped over the same period and increased thereafter.

IV.5 Discussion

Since the main objective of this study was to detail changes in gonadal morphology during sea bream natural sex reversal and correlate these with the sexual endocrine profile, individual tagging of 2-year old fish kept under controlled conditions would have been preferable to the chosen strategy of random seasonal collection from fish-farms. Nevertheless, seasonal collection provided full characterization of the gonads that would have been impossible otherwise. Unfortunately, fish keeping facilities were unavailable at the time and the maximum commercial weight (available all year round) was 250-300g, which corresponds to 16-20 months of age. These fish were experiencing their first spawning season or about to experience their second. Early investigations of sea bream hermaphroditism (D'Ancona, 1941; Pasquali, 1941) reported that sex reversal in wild animals occurs 1 and occasionally 2 years after the initial maturation as a male that takes place at the end of the second year of life, i.e., during the third spawning season. Subsequent studies carried out with captive animals, however, demonstrated that sex reversal could occur earlier. Using individually tagged fish under natural photoperiod, Kadmon *et al* (1985) noticed that 13 out of 50 sex reversals occurred in the second spawning season (and that 24 out of 35 occurred in the third spawning season) while Happe and Zohar (1988)noticed 4 out of 10.

In the present study fish never reached the second spawning season since they were all under 2 years of age. Also, since the fish sampled were not from the same cohort the results may affect gonadal evolution, and since the fish were approximately the same age and weight, it is more the effect of season on 1^{+}year class gonads. Having said that there appeared to be a continuous change in gonad morphology, suggests that the qualitative changes may be representative of what occurs in older fish. Gonadal histology showed that the onset of testicular maturity starts in August to reach full spermiation in November. In January, after the spawning period, spermatozoa cysts became empty, testis began to shrink and early signs of testicular disorganization such as short and irregularly outlined seminiferous lobules with scattered spermatogonia and abundant connective tissue (immune cells?) appeared. According to Bruslé-Sicard (1997), in sea bream, these signs, together with lack of spermatogonial renewal, should be used as criteria for determining the onset of sex inversion rather than simple oogenic activity. Also, in April/May when testicular indexes were at their minimum, some fish exhibited large masses of undifferentiated tissue, together with connective cells, which replaced the spermatogonia and had a yellow-brown appearance after H & E staining. These bodies have not previously been reported in the literature on sea bream. However, they are reminiscent of the "large foci of testicular degeneration" referred to other protandric sparids such as, *Diplodus sargus* or *Acanthopagrus schlegeli*. They were described as well developed networks of collagen fibers with deposition of "yellowish" pigment (Micale and Perdichizzi, 1994) or simply as "yellow-brown bodies" (Chang *et al.*, 1997). Thus, it is possible that some of the fish caught in March-April/May showing these clear signs of testicular regression, i.e. "yellow-brown" bodies, connective tissue spreading all over the testicle and lobular disorganization in a shrinking testicular tissue, were, in fact, starting their sexual inversion. However, we are cautious to make a definitive conclusion because the sea bream sexual plasticity is considerable and cases have been reported of males in which a gonadal biopsy demonstrated numerous non-motile spermatogenic cells and subsequent biopsies only one month later contained post-vitellogenic oocytes (Kadmon *et al.*, 1985). The ovary, despite its higher area in cross sections and the fact that pre-vitellogenic oocytes seemed to evolve in size and developmental stage, did not grow significantly during this period. Similarly, when sea bream gonads were artificially feminized, the effect of exogenous estrogens

(which was expected to "feminize" the gonads) was largely to inhibit testicular growth and male germ cell development beyond spermatogonia stage rather than stimulate ovarian growth (Condeça and Canario, 1999). It is possible, thus, that ovarian growth might happen only in a second stage of sex inversion.

A remarkable observation in the present study was the occasional widely divergent results obtained in the two fish farms. The differences observed in GSI and gonad morphology as well as some minor differences in steroid profiles are possibly a result of the sexual plasticity of this species and of the large intervals between sampling dates. The main environmental factor identified in aquaculture practice that differed between the two fish farms was the rearing density, 1 and 3 fish/m^3, respectively in Maresa and Aquamarim. The large difference in RIA results in plasma 17,20αP between fish farms, however, suggests that rearing density may influence sea bream sexual endocrine patterns. An effect of rearing density on steroid levels has been shown in several teleosts including *Chelidonichthys kumu*, where confinement experiments at different densities affected plasma levels of E_2 and T (Clearwater and Pankhurst, 1997). This could also partially explain the failure to correlate statistically the seasonal steroid levels with gonadal morphology and cytology. Therefore, assuming that the testis has a dominant role in the first stages of sex reversal (chapter II), our attention focused on the periods when the testis showed atrophy (April-May) and at the onset of spermatogenesis (August) or spermiation (September). During this period most androgens (T, 11KT, 11βT) and estradiol-17β assayed, however, were at either low levels in blood or did not show any significant relationship to gonad morphology.

17,20βP is now widely accepted as the maturation-inducing steroid (MIS) in most teleost species (Scott and Canario, 1992; Nagahama, 1997) but this role has also been suggested for another closely related 20β-pregnane, 17,20β,21P, in the sea bream (*Sparus aurata*, Canario *et al.*, 1995), in the Atlantic croacker (*micropogonias undulatus*, Trant *et al.*, 1986) and in the spotted seatrout (*Cynoscion nebulosus*, Thomas and Trant, 1989). 17,20βP can also be synthesized by teleost spermatozoa (Barry *et al.*, 1990a; Ebrahimi *et al.*, 1995; Ebrahimi *et al.*, 1996; Lee *et al.*, 1998) and its seasonal profile is closely

associated with male spawning in salmonids (Scott *et al.*, 1984; Miura *et al.*, 1992; Antonopoulou *et al.*, 1999) in relation to the acquisition of sperm motility (Nagahama, 1994). In Perciformes, higher levels of 17,20βP during spermiation is uncommon in gonochoristic fish (e.g. *Morone* genera, Mylonas *et al.*, 1997a; Mylonas *et al.*, 1997b) and rare in hermaphrodites (e.g. protogynous *Pagrus auratus* and *Thalassoma duperrey*, Hourigan *et al.*, 1991; Carragher and Pankhurst, 1993). In this study neither 17,20βP nor 17,20β,21P were related to spermiation or oocyte development (from chromatin-nucleolar to perinucleolar stage). Surprisingly, the levels of these hormones, which were relatively high in plasma, tended to be significantly higher during the post-spawning period (17,20βP), or during April-August when spermatogonia were prevalent (17,20β,21P), than during spermiation. Results from chapter III showed that sea bream gonads are able to produce 17,20βP, as well as 17,20β,21P . A possible explanation for the low levels during the spawning period is an increased 5β-reductase activity which would lower the level of these steroids (chapter III Vermeirssen *et al.*, 1998). The high levels outside the spawning period can be due to the possibility that, as reported for the rainbow trout, nonflagellated male germ cells have 20β-HSD activity and are able to synthesize these steroids (Vizziano *et al.*, 1996).

The literature largely highlights the importance of 11-oxoandrogens in the teleost male reproductive cycle. Table 4.4 emphasises the species wide ability of male gonads from teleosts (only the first 4 orders are not within the teleosts) to synthesize *in vitro* these steroids and also their systematic presence in blood. Despite the obvious differences in sampling strategies carried out in different studies, only 13 out of the 45 species considered (where T levels were known) had higher T levels than the major 11-oxo-androgen and from these, in 6 cases 11KT was the sole 11-oxoandrogen assayed. Generaly, higher 11KT plasma levels occur before or during the spawning season (see Table 4.4). Antisera availability and the enormous amount of information on the behavioural and physiological importance of 11KT in male germ cell differentiation (e.g. Miura *et al.*, 1994; Miura *et al.*, 1996) are frequently the reasons for only monitoring 11KT. Idler *et al* (1976) were the first to emphasise that in ambisexual species 11βT predominated over 11KT and also that 11βT plasma level in these species was much higher than in gonochoristic species. Since then,

11βA was found to be a major 11-oxoandrogen synthesised *in vitro* in several species including protandric hermaphrodites (*Pagellus acarne* (protandric), Reinboth *et al.*, 1986; *Clarias gariepinus*, Schoonen *et al.*, 1987; *Gasterosteus aculeatus*, Borg *et al.*, 1989; *Lates calcarifer* (protandric), Guiguen *et al.*, 1995) and in the male testis of *Epinephelus microdon* (Debas *et al.*, 1990) and *Lates calcarifer* (Guiguen *et al.*, 1993). Previous *in vitro* results with sea bream gonads have shown that at the onset of spermatogenesis 11βA was a major metabolite from 17P whereas 11βT was not identified and 11KT was present at very low yields (chapterIII). Although a stimulatory action of 11βA in the germinal epithelium, on spermatogenesis, on the development of the seminal vesicle and on the expression of secondary sex characters in African catfish has been described (Resink *et al.*, 1987b; Schoonen *et al.*, 1987) the role of 11βA in sea bream required further investigation. In the present study, the major plasma androgen found was 11βA while the remaining 11-oxoandrogens were practically inexistent or near the detection limit. Yields obtained by 11βA differed considerably between fish collected from the two fish farms. However, in another group of fish, individual profiles of 11βA taken every week from July to November have shown that maximum levels of 11βA were present in September-October at the onset of spermiation (Vília & Canario, unpublished data). Therefore the absence of the 11βA peak in Maresa was most likely due to the large sampling intervals. Thus, considering the high levels present and the steroidogenesis information it is possible that 11βA is important in spermiation. The reason for the March-April peak, however, remains unclear. The importance of 5β-reduction in relation to plasma levels was not considered in this study but in light of the high 5β-Red activity found *in vitro* (chapter III) and the *in vitro* synthesis of 11-oxo-5β-reduced androgens detected by GC-MS as well as the high "5β3α" immunoactivity detected in August and November plasma samples (this study), which had low polarity on RPTLC characteristic of 11-oxo-steroids, it can be inferred that it may have a large influence on the levels of active steroid measured in the plasma and may control their availability at appropriate times and tissues as suggested for other steroids (Kime, 1990).

Table 4.4 – 11-oxoandrogens produced *in vitro* by teleost gonads or detected in blood plasma by RIA. Notes: *In Vitro*- numbers within round parentheses (11-oxo-androgens) or within square parentheses (T) indicate order in magnitude of synthesis; (x)h indicates maximum n° of incubation hours; ... indicates already mentioned steroid synthesis. *In vivo* - number within square parentheses indicates maximum level of T in $ng.ml^{-1}$ for the species. *Sexual stage* without parentheses indicates the period of maximum level of the major 11-oxoandrogen and within parenthesis indicates the period studied (if specified and different). Abbreviations: PG-protogynous; PR-protandric: SH- simultaneous hermaphrodite.

	IN VITRO				*IN VIVO*			
Group/Species	Sexual Stage	Precursor	11-oxo-androgen	Reference	Sexual Stage	11-oxo-androgen	Quantity	Reference
Carcharhiniformes								
Scyliorhinus canicula	Mature	No precursor	11KT (2) [1] 14h (RIA, very low cont.)	(Sourdaine *et al.*, 1990)				
Squaliformes								
Squalus acanthias	Fish caught in the wild in September and May	Progesterone pregnenolone	None. [1] 16h Carriers incl. 11-oxo-and + no unknowns cited Inferred lack of 11β-Hyd	(Simpson *et al.*, 1964)				
Acipenseriformes								
Acipenser gueldenstaedti	Various stages of sexual cycle	Pregnenolone Androstenedione	None. [1] 12h Concluded lack of 11β-Hyd	(Bukovskaya *et al.*, 1997)	Pre-spawning (whole year)	11KT	<110 ng/ml [85]	(Bukovskaya *et al.*, 1997)
Bester (hybrid) (*Huso huso* x *Acipenser ruthenus*)	Various stages of sexual cycle	Pregnenolone 17α-progesterone Testosterone	11KT (RIA)	(Amiri *et al.*, 1999)	Mid-late spermatogenesis (whole year)	11KT	<120 ng/ml [40]	(Amiri *et al.*, 1996)
Ceratodontiformes								
Neoceratodus forsteri	Whole year (4 months sampled)	Progesterone 17α-progesterone Testosterone	None. [1] 3h Concluded lack of 11β-Hyd activity	(Joss *et al.*, 1996)				
Clupeiformes								
Clupea harengus pallasi					Spermiogenesis (Prior and until ripe milt production)	11KT	<280 ng/ml (free+gluc.) [300]	(Carolsfeld *et al.*, 1996)
Anguiliformes								
Anguilla anguilla	Silver stage (treated with hCG)	Androstenedione (Progesterone)	11βA (1) [nd] 2h 3α,11βA5β-17one (2) adrenosterone (3) A5β-3,11,17-trione (3) 11βT (4)	(Eckstein *et al.*, 1980) (steroid yields inferred)				
	Silver stage	Progesterone	11KT (?)	Leloup-Hatey *et al* (1983) *in* (Fostier *et al.*, 1983)				
Anguilla japonica	?	Pregnenolone	11KT [?]	(Miura *et al.*, 1994)	Spermatogenesis (treated with hCG)	11KT	<8 ng/ml [?]	(Chiba *et al.*, 1997)
Anguilla australis					Mature (migratory, "silver")	11KT	<51 ng/ml [1.2]	(Lokman and Young, 1998)
Anguilla dieffenbachii					Mature (migratory, "silver")	11KT	<28 ng/ml [1.2]	(Lokman and Young, 1998)
Salmoniformes								
Salmo gairdneri	Spermiation (various stages of sexual cycle)	17α-progesterone (and progesterone)	11KT (1) [3] 11h 11βT (2) 11βA (4) adrenosterone (5)	(Dépêche and Sire, 1982)	maturing	11KT 11βT 11βA adrenosterone	<27 ng/ml [43] <5 ng/ml <11 ng/ml <9 ng/ml	(Schulz, 1985)
Salmo trutta					Spermiation	11KT	<76 ng/ml (free+gluc.)	(Kime and Manning, 1982)

Group/Species	*IN VITRO*				*IN VIVO*			
	Sexual Stage	Precursor	11-oxo-androgen	Reference	Sexual Stage	11-oxo-androgen	Quantity	Reference
Salmo salar	spermiation	Androstenedione (dehydroepian-drosterone)	11βT (1) [2] 5.5h 11KT (3) 11βA (4)	(Idler *et al.*, 1968)	(whole year) ?	11βT 11KT 11βT	<10 ng/ml (free+gluc.) <222 ng/ml [?] <0.13 ng/ml	(Idler *et al.*, 1976)
Salvelinus alpinus					Initial spawning (whole year)	11KT	< 50 ng/ml [50]	(Tveiten *et al.*, 1998)
Salvelinus fontinalis	Beginning of spermiation and spermiation	Pregnenolone	11βT 11KT	Sangalang & O'Halloran (1973) *in* (Fostier *et al.*, 1983)	Functional maturity (october) (June-December)	11KT	< 27 ng/ml [3]	(Sangalang and Freeman, 1974)
Oncorhynchus kisutch					Pred. spermatids and spermatozoa (Final maturation ; Sep - Nov)	11KT	< 180 ng/ml [70]	(Fitzpatrick *et al.*, 1986)
Oncorhynchus nerka					Silvered colored stage (spawning cycle)	11KT	< 125 ng/ml [100]	(Liley *et al.*, 1993)
Oncorhynchus mykiss								
Oncorhynchus rhodurus	Spermatogenesis and spermiation	No percursor	11KT (RIA) [?] 18h	(Sakai *et al.*, 1989)				
Esox lucius	Spawning	Progesterone (pregnenolone; T)	11KT (1) [3] 3h 11βT (2)	(Kime and Hews, 1978)				
Siluriformes								
Clarias gariepinus	Breeding (Various stages of sexual cycle)	Pregnenolone	11βA (1) [3] 11KT (4) 11βT (2)	(Resink *et al.*, 1987a)	Mature (12-15 months of age)	11KT 11βA 11βT adrenosterone	<7 ng/ml (GC-MS) [17] <2.5 ng/ml <1.5 ng/ml <1 ng/ml	(Vermeulen *et al.*, 1994)
Silurus glanis	spermiation	17α-progesterone	11KT (1) [2] 3h 11βT (3)	(Kime *et al.*, 1993)	Before spermiation	11KT 11βT	<7.5 ng/ml [12, free+glu] <2.5 ng/ml	(Kime *et al.*, 1993)
Brachydanio rerio	?	Pregnenolone or androstenedione	11βA (?) [?] ?h 11KT (?) adrenosterone (?)	(Lambert *et al.*, 1986)				
Cypriniformes								
Cyprinus carpio	spermiation	No precursor	11KT (1) [2] 18h (RIA)	(Barry *et al.*, 1989)	Spermiation	11KT	< 26 ng/ml [12]	(Barry *et al.*, 1990b)
Carassius auratus	spermiation	17α-progesterone	adrenosterone (1) [4] 11KT (2) 11βA (3)	(Kime and Scott, 1993)	Spermiation	11KT	< 13 ng/ml [7]	(Kobayashi *et al.*, 1986)
Rutilus rutilus	spermiation	17α-progesterone	11βA (1) [4] 1-18h 11KT (2 adrenosterone (3)	(Ebrahimi *et al.*, 1995)				
Catostomus commersoni					Prespawning (spawning migration)	11KT	< 80 ng/ml [15]	(Scott *et al.*, 1984)
Cyprinodontiformes								
Poecilia latipinna	? Fish with 3.5-5.5gr body weight (viviparous)	Testosterone	3β,11β,17βA5β [nd] 3h	(Kime and Groves, 1986)	Mature (mature and regressed)	11KT 11βT	<2 ng/ml [5] <1.5 ng/ml	(Kime and Groves, 1986)
	?	Androstenedione	11βA 11KT 11βT	Van Den Hurk & Lambert (1977) *in* (Fostier *et al.*, 1983)				
Fundulus heteroclitus					Breeding season (whole year)	11βT 11KT	<30 ng/ml [14] <11 ng/ml	(Cochran, 1987)
Jenynsia lineata	Immature (viviparous)	Pregnenolone (or progesterone)	11βA (1) [3] 4h adrenosterone (2)	(Tesone and Charreau, 1980)				
Mugiliformes								
Mugil cephalus	Spermiation	Progesterone or pregnenolone	none	Eckstein & Eylath (1968) *in* (Fostier *et al.*, 1983)				
Synbranchiformes								
Monopterus albus (PG)	Male phase	Testosterone	11KT (1) [prec.] 2h Adrenosterone (2)	(Chan and Yeung, 1989)	Male phase Spawning (includes inactive and prespawning periods)	11βT 11KT	<0.2 ng/ml [0.25] <0.1ng/ml	(Chan and Yeung, 1989)
Gasterosteiformes								

Group/Species	*IN VITRO*				*IN VIVO*			
	Sexual Stage	Precursor	11-oxo-androgen	Reference	Sexual Stage	11-oxo-androgen	Quantity	Reference
Gasterosteus aculeatus	Breeding	Pregnenolone (or androstenedione)	adrenosterone (1) [nd] 3h 11βA (2) 11KT (3)	(Borg *et al.*, 1989)	Breeding (11KT) and December (adrenosterone and 11βA) (whole year)	Adrenosterone 11KT 11βA 11βT	<30 ng/ml (December) [5] <25 ng/ml (July/breeding) <18 ng/ml (December) <3 ng/ml (May)	(Mayer *et al.*, 1990)
Perciformes								
Perca fluviatilis	Breeding season	Testosterone	11βT (1) [prec.] 3h 11KT (2)	(Kime and Hews, 1978)				
Stizostedion vitreum					Pre-spawning (whole year)	11KT	<40 ng/ml [3]	(Malison *et al.*, 1994)
Lepomis macrochirus					Spawning	11KT	<34 ng/ml (parental) [5] <1.5 ng/ml (sneakers) <1 ng/ml (satellites)	(Kindler *et al.*, 1989)
Spicara maena (PG)	Spawning	Testosterone	11βT (1) [prec.] ?h 11KT	(Reinboth, 1979)				
Tilapia zillii *Oreochromis aureus* *Sarotherodon mossambicus*	Mature. Spermiation	Testosterone	3α17βA5β-11one (1) 11KT (2) [prec.] 3h 11βT (3)	(Kime and Heyder, 1983)	Mature / one year old September (May-September) Mature. Spermiating	11KT 11KT 11βT 11KT	<12 ng/ml (territorial) [5] <15 ng/ml (territorial) [18] <5.6 ng/ml [18] <5.4 ng/ml	(Neat and Mayer, 1999) (Mol *et al.*, 1994) (Kime and Heyder, 1983)
Betta splendens	Adult	Progesterone	11KT (1) [5] 2h 11βA (2) 11βT (3) adrenosterone (4)	(Leitz and Reinboth, 1987)				
		Pregnenolone	..3β11βA5α 11β17βA5β (+ metab.)					
Lates calcarifer (PR)	Spermiation	Androstenedione	11βA (1) [4] 1h	(Guiguen *et al.*, 1995)	Male phase. Sex inversion (whole year)	11KT 11βA	<0.5 ng/ml [0.6] <8 ng/mg (gonad content)	(Guiguen *et al.*, 1995) (Guiguen *et al.*, 1993)
	Transitional stage I (to female)	Androstenedione	..adrenosterone (2) 11KT (3) 11βT (5)					
Sparisoma viride (PG)					Male phase (territorial and non-territorial)	11KT	<1.5 ng/ml (territ.) [2.5] <1 ng/ml (non-territ.) [2]	(Cardwell and Liley, 1991)
Amphiprion melanopus (PR)					Male phase	11KT	<0.7 ng/ml [1.5]	(Godwin and Thomas, 1993)
Acanthochromis polyacanthus					Spawning higher peak (November) (various phases of gonadal develop Paternal caring fish)	11KT	<1.2 ng/ml (territorial) [2.2] <0.9 ng/ml (non-territ.)[1]	(Pankhurst *et al.*, 1999)
Chromis dispilus					Fully spermiated, (territorial and non-territorial)	11KT	<25 ng/ml (territorial) [5] <21 ng/ml (non-territ.) [5]	(Barnett and Pankhurst, 1994)
Hypsypops rubicundus					Pre-mating (Brood cycle. Paternal caring fish)	11KT	<24 ng/ml [15]	(Sikkel, 1993)
Thalassoma duperrey (PG)	Terminal (primary and secondary) phase males (all male phases, incl. initial phase)	17α-progesterone (Testosterone)	11KT (1) [3] 18h 11βT (2) 11βA (3)	(Hourigan *et al.*, 1991)	Terminal phase male (various phases of sex change)	11KT	<4 ng/ml [0.6]	(Nakamura *et al.*, 1989)
Coris julis (diandric PG)	Primary and secondary males	Testosterone	11βT (1) [prec.] 11KT (2) 11βA (3) adrenosterone (4)	(Reinboth and Becker, 1984)				
Glossogobius olivaceus	Breeding season	Progesterone Pregnenolone 17α-progesterone Androstenedione testosterone	None Concluded lack of 11β-Hyd activity	(Asahina *et al.*, 1985)				
Gobius jozo	Breeding season	Pregnenolone	11βA (?) [?] 6h	(Colombo *et al.*, 1977)				
Gobius paganellus	Breeding season	Pregnenolone	None [1] 4h	(Colombo *et al.*, 1970)				
Gillichthys mirabilis	Breeding season	Progesterone	11βA (1) [2] 6h	(Colombo and Beldevere, 1977)				
Dicentrarchus labrax	Spermiation	Pregnenolone	11KT (1) [4] 6h 11βA (2) 11βT (3) adrenosterone (5)	(Colombo *et al.*, 1978)	Spermiation (whole year)	11KT	<7 ng/ml [14]	(Prat *et al.*, 1999)
Morone saxatilis	Spermiation	Progesterone	11βA (1) [2] 6h	(Colombo and Beldevere, 1977)	Spermiation	11KT	<5 ng/ml [3]	(Mylonas *et al.*, 1997b)

Group/Species	*IN VITRO*				*IN VIVO*			
	Sexual Stage	Precursor	11-oxo-androgen	Reference	Sexual Stage	11-oxo-androgen	Quantity	Reference
(former *Roccus saxatilis*)								
Morone chrysops								
Serranus cabrilla (SH)	Male tissue only	Testosterone	11βT (1) [prec.] ?h 11KT (2) 11βA (?)	(Reinboth, 1979)	Spermiation ambisexual stage Juveniles approaching maturity	11KT 11KT 11βT	<1.8 ng/ml [0.8] n.d. [?] <15 ng/ml	(Mylonas *et al.*, 1997a) (Idler *et al.*, 1976)
Epinephelus morio (PG)								
Epinephelus microdon (PG)								
Epinephelus tauvina (PG)	Male phase (androgen induced)	Testosterone	11βT (2) [1] 30h 11KT (3)	(Lee *et al.*, 1995)	Male phase. Breeding (whole year) Male phase (whole year)	11KT 11KT 11βA	<6 ng/ml [4] <8 ng/ml [5] <0.5 ng/mg (gonad cont.)	(Johnson *et al.*, 1998) (Debas *et al.*, 1990)
Centropristis striatus								
Sparidentex hasta (PR) (former *Acanthopagrus cuvieri*)					Male phase. Breeding (whole year) Male phase. Spawning (whole year) Male phase. Spawning (whole year) Male phase. End of spawning season (flowing sperm still present) Male phase. Approaching spawning season	11KT 11βT 11KT 11βT 11KT 11KT 11βT 11KT 11βT	<4.5 ng/ml [0.25] nd <2.5 ng/ml [1] <1.2 ng/ml <0.8 ng/ml [0.2] n.d. [?] <11 ng/ml n.d. [?] <19 ng/ml	(Cochran and Grier, 1991) (Kime *et al.*, 1991) (Chang *et al.*, 1997) (Chang *et al.*, 1994) (Idler *et al.*, 1976) (Idler *et al.*, 1976)
Acanthopagrus schlegeli (PR)								
Diplodus sargus (partial PR)								
Pagellus acarne (PR)	Breeding season	Androstenedione	11βA (1) [2] 2h 11βT (3) adrenosterone (3) 11KT (4)	(Reinboth *et al.*, 1986)				
Pagellus erythrinus (PG?)								
Pagrus auratus (partial PG)					Male phase. Beginning of spawning season Spawning season	11KT 11βT 11KT	n.d. [?] <7 ng/ml <3.5 ng/ml [1.5]	(Idler *et al.*, 1976) (Carragher and Pankhurst, 1993)
Rhabdosargus sarba (PR)	Male phase. Spawning (various stages of sexual cycle)	Testosterone	11βT (1) [prec.] 2h 11KT (2)	(Yeung and Chan, 1985)	Male phase. Spawning (including inactive and prespawning periods)	11βT 11KT	<0.25 ng/ml [0.4] n.d.	(Chan and Yeung, 1989)
Pleuronectiformes								
Microstomus kitt	Mature	Androstenedione Testosterone	11βA (?) [?] ?h adrenosterone 11βT 11KT	(Simpson *et al.*, 1969)				
Pleuronectes platessa								
Pleuronectes vetulus					Spermiating	11KT	<25 ng/ml (free+gluc.) [10]	(Vermeirssen *et al.*, 1998)
Pseudopleuronectes americanus					Spawning (Spawning and gon. recrudescense) ?	11KT 11KT 11βT	<5.5 ng/ml [1] <12 ng/ml [?] <0.12 ng/ml	(Sol *et al.*, 1998) (Idler *et al.*, 1976)
Hippoglossus hippoglossus					Before sperm release (whole year)	11KT	<20 ng/ml [10] (1 fish)	(Methven *et al.*, 1992)

The high immunoactivity of the "5β3α" antisera on RPTLC separated plasma fractions confirmed *in vivo* that 5β-reduced steroids are abundant in sea bream blood plasma. The cross-reactivities of related "5β3α" co-migrating steroids were relatively high but far less than 100%, which suggests that the real level of such steroids in fish blood plasma must be very high. For example, the peak closest to the origin migrated near the elution position of 3α,17,20αP5β and co-migrated with 3αA5β standard, which were both synthesized *in vitro* and cross-reacted , respectively, 44% and 9% with the antisera. Therefore, it can be estimated that several ng of unknown steroid was present. If the steroid was mainly 3αA5β then its real level could be up to 20 $ng.ml^{-1}$. For the second peak nearest to the origin, several "5β3α" steroids with a C21 hydroxyl group and high cross-reactions with this antiserum are known to co-migrate, e.g. 3α,17,20β,21P5B (100% cross-reaction), 3α,17,20α,21P5β (cross-reaction not tested) and 3α,17,21P5β (64% of cross-reaction). The major "5β3α" peak (the third from the origin) was isopolar with the larger peak detected by the "1720αP" antisera suggesting a steroid reacting with both antisera. However, more than one steroid must be present since the immunoactivity profiles with both antisera varied in a non-parallel fashion at different sampling dates. Candidate steroids with significant cross-reactions include 3α,11β,17,20αP5β (25% cross-reaction) and 3α,11β,17,21P5β (16% cross-reaction), both synthesized *in vitro* in this study. The latter steroids are, however, related to cortisol, which is known to be rapidly produced in fish undergoing stress (Jones and Mosley, 1980) such as high rearing density (Tort *et al.*, 1996; Auperin *et al.*, 1997), or capture and handling of fish during sampling (Clearwater and Pankhurst, 1997). Independently of the specific configuration of these steroids, 5β-pregnanes may have a physiological role in spawning males. In the male plaice *Pleuronectes platessa*, gonadotrophin-releasing hormone agonist (GnRHa) stimulated milt volume and fluidity via increased plasma concentration of "5β3α" steroids (Vermeirssen *et al.*, 1998). In the male grouper, *Epinephelus coioides*, 5β-pregnanes accounted for 75% of total product yield from spermatozoa incubation with labelled 17P (Lee *et al.*, 1998). Further studies are required to determine de nature of these steroids.

The striking difference between the "17,20αP" plasma levels in fish from the two fish farms, however, remains unclear. The cross-reaction of the antiserum with metabolites related to cortisol is very small (< 0.1%; at least with the steroids tested in this study) and thus it seems unlikely that the higher rearing density in Aquamarim could explain the higher "17,20αP" immunoactivity in this fish farm.

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CHAPTER V

EFFECT OF ESTROGEN ADMINISTRATION DURING EARLY DEVELOPMENT OF THE GONADS OF SEA BREAM, *Sparus aurata* (L.)

V Effect of estrogen administration during early development of the gonads of sea bream, *Sparus aurata* (L.)

V. 1 Abstract

Sea bream were raised from hatch to the first functional spawning season and submitted to feeding regimes, in which estradiol-17β (E_2) was added to the food supply by either incorporation in rotifers and artemia or spraying onto commercial diet, at different periods of their life cycle. Estrogen uptake by rotifers and artemia resulted in an estrogen larval content superior to the amount estimated as sufficient for sex reversal in adult sea bream. Sea bream larvae appeared to be insensitive to estrogen exposure during the first 100 days of life. A gonadal *primordium* was detected by day 87 in both estrogen-treated or control larvae and all fish became functional males at spawning. The reasons for this apparent estrogen insensitivity are discussed.

KEY WORDS: sex reversal, gonad differentiation, estradiol-17β, larvae, juvenile

Running title: Effect of estrogen during early development of the gonads

V. 2 Introduction

The mechanisms of sex differentiation and sex reversal are still largely unknown in fish but steroids have been shown to have a direct effect to masculinize (androgens) or feminize (estrogens) fish gonads (see review Baroiller *et al.*, 1999). The importance of timing of such hormonal treatments was first emphasized by Yamamoto (1969) who stated that they should start when gonads are still undifferentiated and be continued until after sexual differentiation. Subsequent experiments carried out mainly with salmonids have shown that in some species timing of treatment could be restricted exclusively to the undifferentiated period (Goetz *et al.*, 1979; Baker *et al.*, 1988). In adult sea bream, exogenous estrogen is capable of inducing precocious sex reversal (Happe and Zohar, 1988; Condeça and Canario, 1999) but it is not known if the same effect is obtained when performed during larval and juvenile stages. In common with sea bream, larval rearing of the lumpfish (*Cyclopterus lumpus*) is improved in survival and growth if live preys are used. Estradiol-17β (E_2) enriched brine shrimp artemia was able to induce 100% sex reversal in this gonochoristic fish larvae whereas direct immersion of eggs in E_2 was rather inconsistent (Martin-Robichaud *et al.*, 1994). It was suggested that estrogen treatment triggered sex reversal before gametogenesis but not before gonadogenesis. In contrast to salmonids, which have gonads at hatching and differentiate soon after (Piferrer and Donaldson, 1993), gonadogenesis in sea bream is thought to occur around 90 days post hatch (Power, D.M., personal observations) and differentiation of the first germ cells occurs at 4 months of age (Zohar *et al.*, 1978). Therefore, a time phased estrogen-feeding scheme for sea bream larvae feminization would be helpful in providing information about the mechanisms of estrogen action on the gonad.

The objective of the present work was to determine the sensitive period of sea bream towards E_2, by administration of E_2 in rotifers, *Artemia*, and a commercial diet, so as to encompass the period of gonadogenesis, and determining the status of the gonads periodically and at the first spawning period.

V. 3 Materials and Methods

Eggs and larvae - Experiments were carried out at CRIP-Sul experimental fish station (Instituto de Investigação das Pescas e do Mar, Olhão, Portugal). Fertilised sea bream eggs were obtained from local spawners. Approximately 40g of floating (good quality) eggs were incubated in 200L conical shape tanks with sterile seawater until hatch. Seawater was sterilised with sodium hypocloride for 5 hours and neutralised with sodium thiosulphate. Fertilised sea bream eggs were raised from hatch to first functional spawning season and submitted to feeding schemes in which control differed from treated fish in that in the latter estradiol-17β (E_2; Sigma-Aldrich Co., Poole, Dorset, UK) was added to the food supply at different periods of the fish life cycle (see Table 5.1). Experiments were started on 20-May-95 (day 0) and ended on 18-September- 96 (day 507).

Larval culture system – The larval culture system consisted of six 200L conical shape tanks among which 9000 larvae were randomly distributed. Seawater was first decanted, filtered, run through a biological filter, warmed to 23 ± 1°C, and delivered to the open circuit system. Water was renewed at 25% of tank capacity per hour, aeration was gentle and photoperiod adjusted to 14 hours light phase. Salinity was 37±1 ‰ during the first 9 days and then reduced to 29±1 ‰. Air traps allowed concentration and removal of fat material. Water outlets were covered with a net of an appropriate pore size for the feeding regime.

During the rotifer phase the outlet net pore size was 80 μm during the day and 150 μm at night to ensure that uneaten rotifers were removed. Approximately 1.2 million rotifers were given to larvae daily aiming to maintain their concentration at 5 per ml since at this stage food concentration is important for successful larval feeding activity. A concentrated microalgae solution (*Chlorella* sp.) was delivered in several small doses to improve rotifer survival in larval tanks. During the *Artemia* phase pore size was 150 μm during the day and 500 μm at night also to allow renewal of uneaten *Artemia*. Approximately 30000 nauplii per tank were delivered daily at the beginning of this feeding phase and increased to nearly 120000 towards the end. The rate of delivery was

decided daily to maintain *ad libitum* a food supply. The daily diet of either rotifers or *Artemia* was divided in four meals.

Rotifer enrichment – Rotifers *(Brachionus plicatilis)* were harvested from stock containers with a 55 μm plankton net and placed in 15L cylindroconical containers for enrichment, with highly unsaturated fatty acids (HUFA) and other essential nutrients necessary for larvae survival not present in unenriched rotifers. Enrichment was performed using 250mg of Protein Selco (*Artemia* systems N. V., Ghent, Belgium) per litre of 20‰ salinity water containing half a million rotifers. Vigorous aeration from the bottom prevented specimen sedimentation. To the estrogen larval diets, rotifer enrichment was complemented with 1ml of E_2 stock (10mg $E_2.ml^{-1}$ ethanol) per litre, i. e., 20mg of E_2 per million of rotifers. Control diet received the same amount of 100% ethanol. Enrichment period lasted 16 hours and was followed by an abundant seawater rinse.

Artemia hatching technique and enrichment – Brine shrimp (*Artemia* sp) cysts AF *Artemia* and EG *Artemia* were purchased from *Artemia* systems N.V., Ghent, Belgium. AF *Artemia* has high HUFA content and small nauplii size (adequate for fish larvae mouth size after the rotifer phase) allowing direct consumption. Therefore, AF cysts were only hydrated, decapsulated and hatched. Hydration was carried out in conical basins with fresh water for 2 hours and using vigorous bottom aeration. Deencapsulation was made using the method of Sorgeloos (1977). Basically, hydrated cysts were placed in stirred salt water and hypochlorite solution kept at pH>10 for 5-15 minutes until the cyst colour became orange. Cysts were rinsed in a 100 μm screen until the chlorine smell disappeared, and hypochlorite was then deactivated with a sodium thiosulphate solution. Decapsulated cysts were hatched in 20‰ saline water.

EG *Artemia* cysts were hydrated, decapsulated and hatched the same way as the AF type, but nauplii from these cysts had to be raised and enriched for 36 or 48 hours to increase size and HUFA content. Enrichment for 36hr and 48hr *Artemia* was carried out in the same manner as for rotifers but only 100000 nauplii per litre were used. Estimates were made assuming that 1g of dry cysts is approximately equivalent to 4g of wet decapsulated cysts and these originate roughly 200000 nauplii. Estimates were checked

under a dissecting microscope before enrichment. For the 36hr *Artemia*, 24hr post-hatch nauplii were submitted to a 15hr enrichment period. For the 48hr *Artemia,* nauplii enrichment was carried out in the same way but included two periods of 15hr separated by an abundant rinse. Protein Selco was used for enrichment at a concentration of 300mg/L and complemented with 1ml of E_2 stock (10mg E_2/ml 100% ethanol) per litre for estrogen diets, i. e., 1mg of E_2 per million *Artemia* nauplii. Control diets had the same amount of 100% ethanol. After enrichment *Artemia* were rinsed with abundant seawater and given to larvae.

Monitoring estrogen uptake – Estrogen contents of rotifers, *Artemia*, and rearing water, was monitored by radioimmunoassay both during enrichment and during the larvae feeding period. Estrogen uptake by larvae was also monitored but only during *Artemia* EG 48hr feeding. Consecutive 300ml samples were taken from the rotifer enrichment container by filtration through a 55μm plankton net. Water was collected in bottles and rotifers thoroughly rinsed with distilled water, wrapped in aluminium paper, before storage at –20°C. Samples were taken immediately after adding the enrichment solution (0 hr) and, 1, 4, 8 and 16 hours afterwards. After enrichment, rotifers were rinsed, filtered and kept in a container with similar conditions to the tanks with sea bream larvae. Samples were taken 1, 2, 4, 8 and 10 hours from these tanks to evaluate loss of E_2. *Artemia* monitoring was carried out in the same way but two enrichment periods were considered. Sampling was made at 0, 2, 8, 15hr (first period) and 24, 26, 28, 30 and 36hr (second period) after the first enrichment. Only one sample was taken 13 hours after *Artemia* release in sea bream larval tanks to evaluate loss of E_2. Larval monitoring was done on individuals from tank G4 and control at day 54 with the feeding scheme ensured by EG 48hr *Artemia* (Table 5.1). Samples were taken in the morning before feeding and after two (late morning) and 4 *Artemia* meals (evening).

Small portions of enrichment water samples (10ml) were paper filtered to eliminate suspensions and then passed through solid phase extraction cartridges from Waters (Sep-Pak Vac 3cc, 500 mg, C18; Millipore corporation, Milford, Massachusetts, USA) for E_2 extraction. Liquid flow through cartridges were maintained constant using a water vacuum pump. Cartridges were activated with 2ml of 100% ethanol (twice) and 2ml of distilled water (twice). After sample flow, another 2ml of distilled water was passed through the cartridges. Elution was made with 2ml of 100% ethanol (twice)

under 800 rpm centrifugation. Ethanol was evaporated in a speedvac concentrator (Savant SC110A; Savant instruments inc., Farmingdale NY 11735) and 1ml of phosphate buffer with bovine serum albumine added before RIA. Solid samples (rotifer, *Artemia* and larvae) were dried at 60°C for 48hr and weighted in pre-weighted Petri dishes. Samples were then eluted in Ringer's solution, sonnicated for 2 minutes, extracted twice with 4ml of diethyl ether and analysed the same way as liquid samples. Tritiated E_2 was used to verify extraction efficiency.

Juvenile culture system – On the 56th day of experiment sea bream larvae were transferred to 1000L tanks and cultured under photoperiod, salinity and water temperature conditions in Southern Portugal. Water was decanted, mechanically filtered and renewed at 10% of tank volume per hour (increasing gradually as fish grew). Dry food (commercial diets) was given *ad libitum* to fish throughout the day using an automatic feeder. Fish feeding observations and quantities of uneaten food at the bottom of the tank from previous day were included in calculations of daily diet estimation. The first diet used was Nippai n°3 (Nippon Formula Feed, co., ltd., Yokohama, Japan) with a particle size of 300-500µm, which is appropriate for initial non-living feed. The second diet was Fry Feed Kyowa B-700 (Kyowa Hakko Kogyo co., ltd., Tokyo, Japan) which had a bigger particle size (700µm) and, afterwards, commercial diets of increasing pellet size were used (Ewos Ltd, Scotland). Direct spraying of an ethanol-E_2 solution over a thin layer of pellets ensured uniform estrogen deposition on commercial diets. Estrogen diets contained 15mg of E_2 per kg and control diets were sprayed with ethanol only.

Feeding design and sampling procedure –Five groups of sea bream larvae (G1-G5) with different feeding strategies and one control group (CTL) were assigned to six tanks. As shown in Table 5.1, G1 received E_2–rotifers between day 4 (larvae mouth opening and first feeding day) and 25, followed by normal (without E_2) *Artemia* and commercial diets. G2 was fed a similar way but the estrogen administration period was extended through the *Artemia* EG 36 and 48hr feeding stage (until larval transfer to juvenile tanks; 56th day). G3 fish received E_2 during *Artemia* EG 36hr + EG 48hr phase, i.e. from day 25 to 56, while G4 had the estrogen period augmented until day 101. G5 fish died just before their intended E_2 feeding period (102nd to 177th day) because of a water supply failure. These problems also occurred with G1, G2 and G3 on different

occasions and as samples had been collected up to this stage, an early gonad evolution was studied in these groups.

The sampling procedure involved sampling of larvae-juveniles at day 87 and 3 other periods at days 298, 430 and 507 (September 18th). Whole larvae or juveniles, or just the gonad of adult specimens, were fixed in Bouin's fixative for 24-36hr, dehydrated and embedded in paraffin wax. Transverse sections were cut (6 μm) and mounted on glass slides and stained with Ehrlich's hematoxylin and eosin.

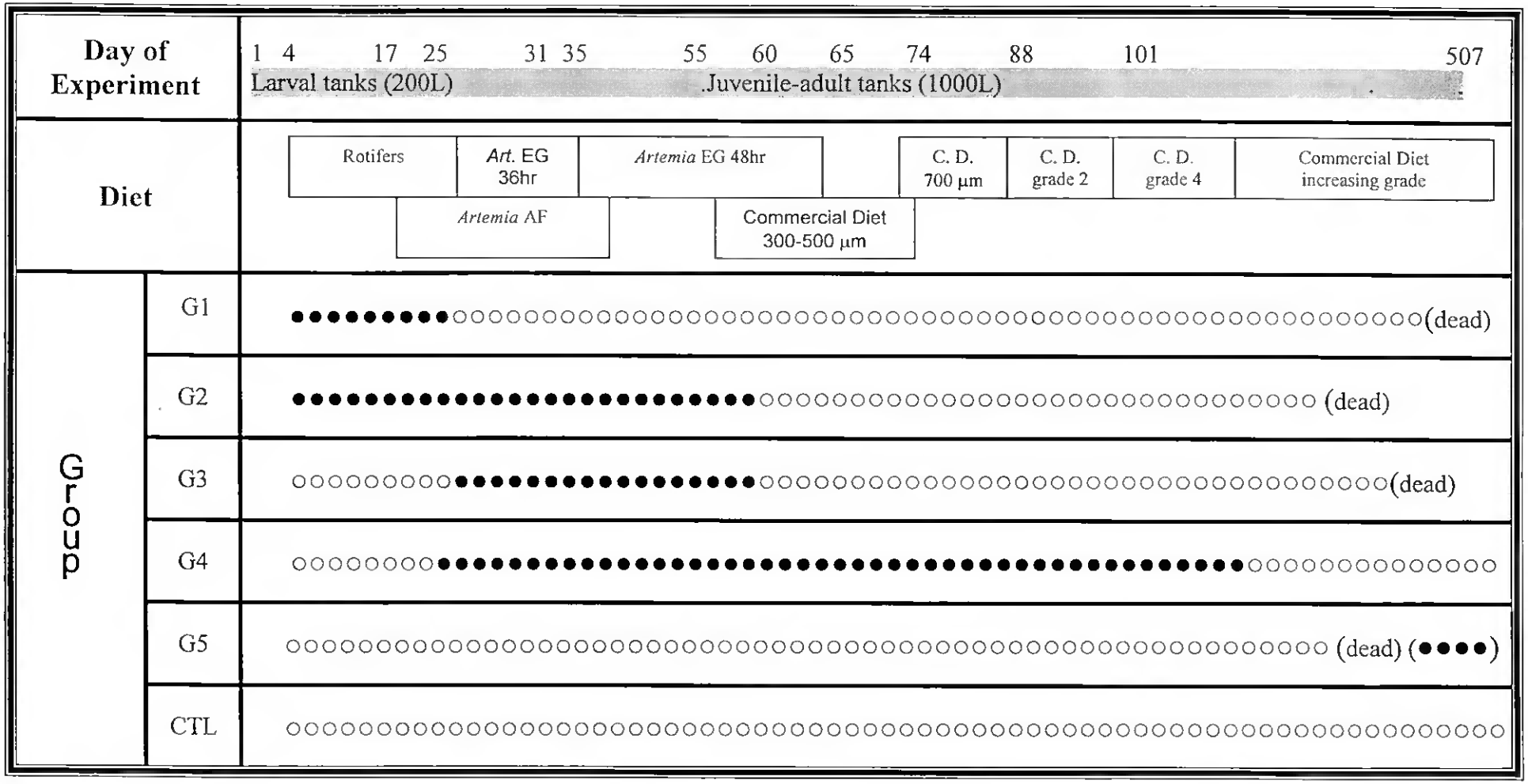
Statistics – Non-linear regressions were used to plot graphs of E_2 content in live prey (see figs. 5.1 and 5.2). Histograms are presented as mean ± SEM. The effect of estrogen on gonadal morphology was tested by two-way Analysis of Variance (ANOVA) followed by Tukey's Honestly Significant Difference test. Data (percentages) was first inverse sine transformed before ANOVA. Plots in Figures are based on untransformed data. Statistical significance was considered at the 5% level.

V. 4 Results

V. 4.1 Rotifer estrogen uptake

Water of rotifers containing estrogen evidenced an initial 1hr period of maximum estrogen content (even superior to estimated initial concentration of 10mg/ml) followed by a continuous decrease until 2.2 mg/ml at the end of enrichment period. After rotifer release in larval tanks there was a slow transfer of E_2 into the larval rearing water which attained 1.1 mg/ml after 10 hr of rotifer presence (Fig. 5.1a). Simultaneously with the rotifer rearing water estrogen decrease, the rotifers E_2 uptake increased continuously up to nearly 1.6 μg per mg by the middle of the enrichment period (8hr) but the rotifer content decreased afterwards to a final level of 0.7 μg per mg of rotifers (Fig.5.1b). After the E_2-treated rotifers were released in larval rearing water, the E_2 content of rotifers remained approximately constant for 2hr but then decreased by 50% (0.4μg per mg of rotifers 4hr after release) and this level remained until the end of the 10hr monitoring period (Fig. 5.1b).

Table 5.1 – Feeding protocol in experimental groups (G1-G5) and control (CTL). Black circles indicate estrogen diet and white circles indicate normal diet. Diet within parenthesis indicates intended estrogen diet not performed because of accidental fish death.



Estrogen levels in either rearing water or body of non-treated rotifers were always negligible. Estrogen extraction efficiency from rotifers was, on average, 60%.

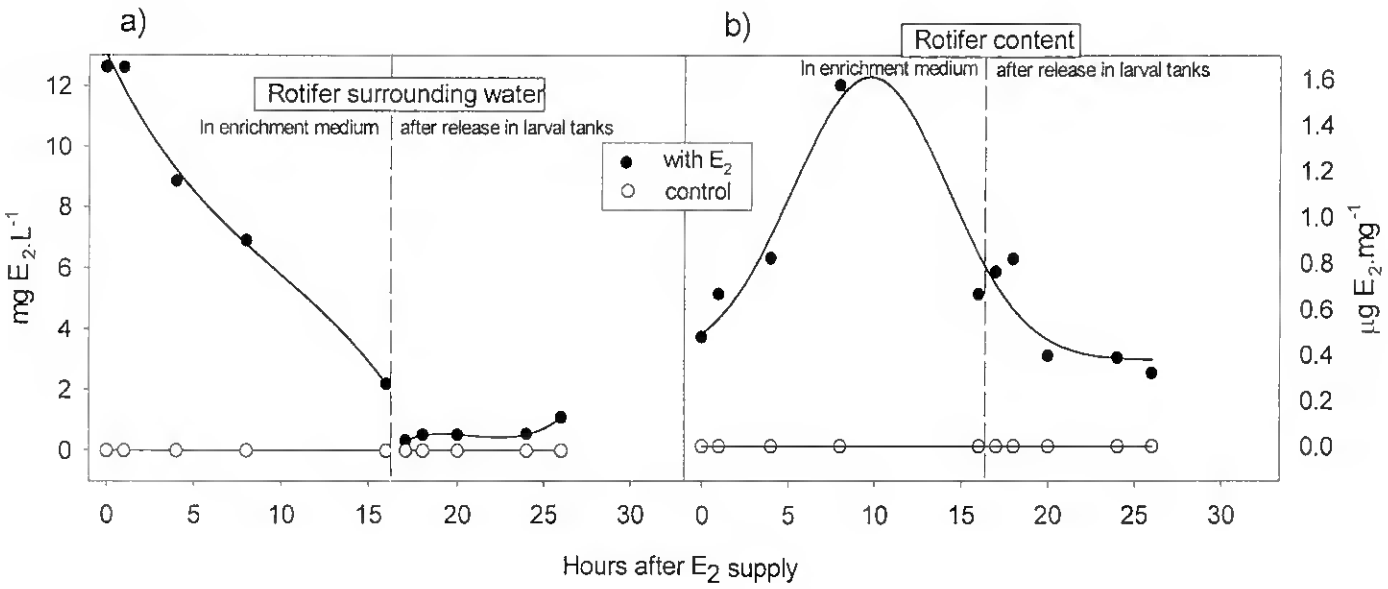


Figure 5.1 – Incorporation of estrogen from enrichment medium (a) into rotifer body (b). The decline in estrogen during the enrichment period was simultaneous with the increase in rotifer estrogen content up to 10hr when it started to decline. The E_2 curves are fitted by non-linear regressions (r^2>0.93 in all cases).

V. 4.2 Artemia estrogen uptake

The level of estrogen in *Artemia* rearing water decreased more sharply than that observed for rotifers, since 1hr after the beginning of the first enrichment period estrogen levels in the water were already 3-fold lower (2.7mg/ml) than the initial E_2 level (8mg/ml) but stabilised thereafter at 2mg/ml until the end of this period. In the second enrichment period, the initial estrogen decrease was compensated by a new E_2 input and decreased afterwards more gradually.

The final *Artemia* E_2 level before release in larval tanks was 4mg/ml (Fig.5.2a). Estrogen release from *Artemia* to larval rearing water was not determined. The uptake of E_2 by *Artemia*, unlike rotifers, was progressive until the end of the two enrichment periods. *Artemia*-E_2 content increased uniformly during the first stage and continued increasing during the second stage in a exponential manner yielding a final level of 1.3

μg E_2 per mg of *Artemia*. Total E_2 in *Artemia* decreased from 1.3 to 0.4 μg 13 hr after its release into larval tanks (Fig.5.2b). Estrogen levels in control experiments in both rearing water or *Artemia* body were always negligible. Estrogen extraction efficiency from *Artemia* was on average 90%.

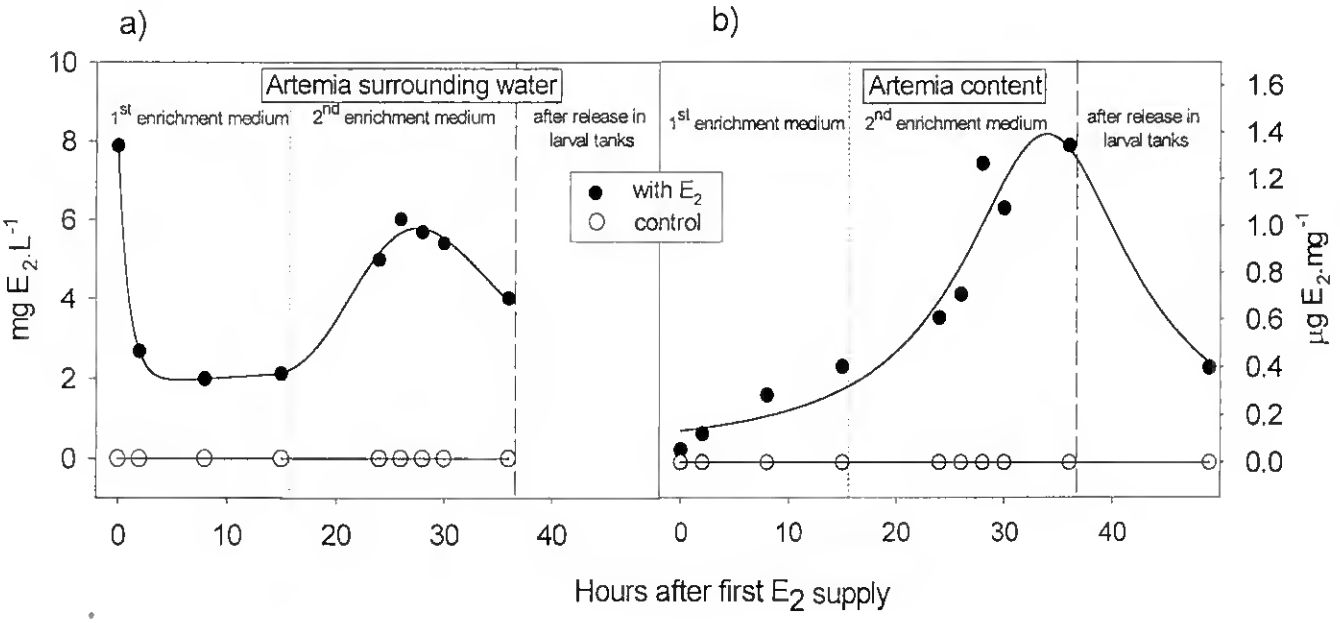


Figure 5.2 - Incorporation of estrogen from enrichment media (a) into *Artemia* body (b). The decline in estrogen in the bathing water during the 1st enrichment period was compensated by the second enrichment period, which permitted a continuous increase in *Artemia* estrogen uptake. E_2 curves are fitted by non-linear regressions (r^2>0.93 in all cases).

V. 4.3 Larval estrogen uptake

Larval monitoring during *Artemia* EG 48hr feeding showed that most estrogen uptake occurs in the 2 morning meals since the 2 evening meals did not contribute to a further net increase in E_2 body content (Fig.5.3), even decreasing by 17% during this period (from 150 to 125 ng/larvae). The E_2 content of fasting larvae in the following day was approximately 1% (less than 2 ng/larvae) of that measured after the second *Artemia*-E_2 meal. Levels of E_2 in control larvae never surpassed 20 pg/larvae. Estrogen extraction efficiency from larvae was on average 90%. Rotifer and commercial diet phases were not monitored.

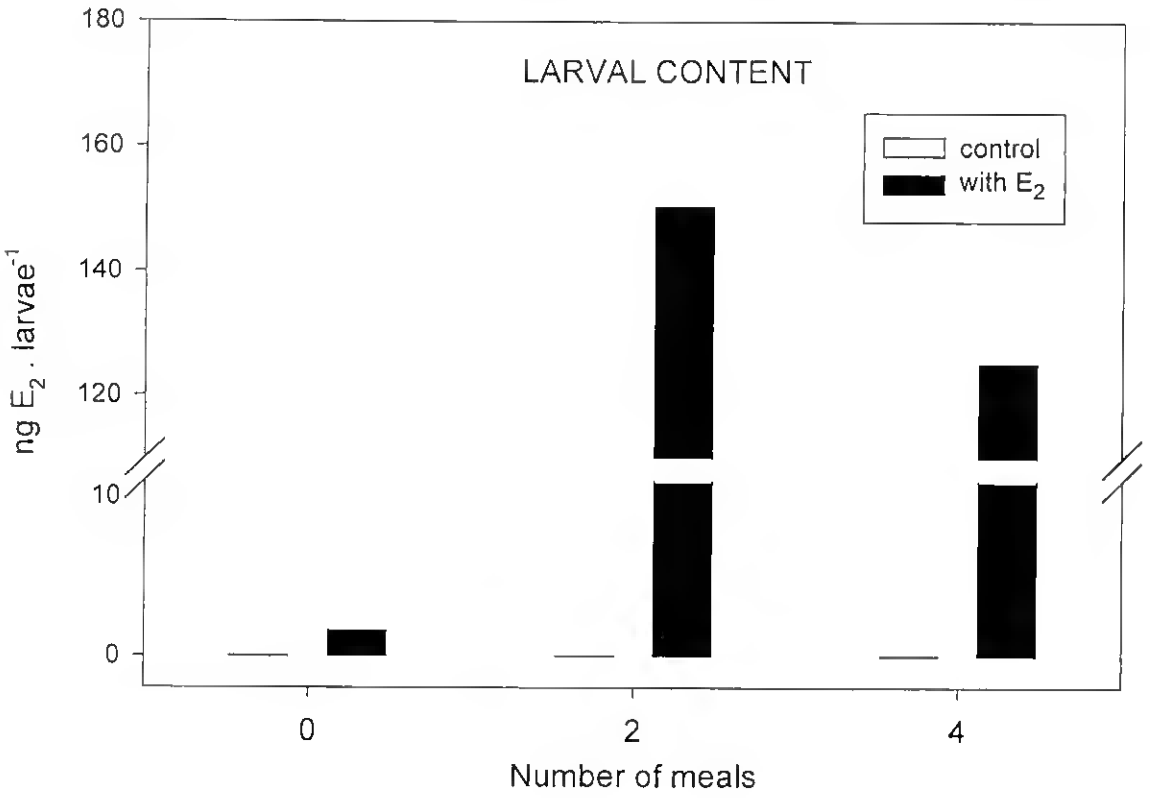


Figure 5.3 – Estrogen body content in larvae during *Artemia* EG 48hr feeding. The main estrogen uptake occurred in the first two morning meals and a 17% decrease in E_2 occurred in the afternoon despite feeding E_2 enriched *Artemia* in further 2 afternoon meals. Fasting larvae (0 meals) contained only 1% of the determined maximum E_2 content measured on the previous day.

V. 4.4 Effect of estrogen on gonadal histology

At day 87 post-hatch, the gonadal *primordium* was visible in both estrogen treated fish and control groups (fig. 5.4a) confirming previous observations (Power, D.M., personal observations). The next sampling period was performed during the juvenile development phase (day 298) and fish from all groups had differentiated gonads, although with scarce germ cells. Thus, it is not possible to make any conclusions about a possible effect of early estrogen treatment on the timing of differentiation of such cells. Preliminary results showed, however, that the first recognizable germ cells are oogonia that arise from primary germ cell nests in the central cavity border around 5-6 months of age (approx. 15-20g body weight; Fig. 5.4b,c,d). According to Zohar *et al* (1978) this event takes place 1-2 month(s) earlier but this difference may be a

consequence of the higher rearing temperatures (21 ± 2 °C all year round) used, since no weight data was shown.

Estrogen appeared to had no detectable effect on adult gonad histology in the surviving fish groups, i.e., G1, G4 (and control) which were exposed to estrogen during the rotifer phase (until day 25) and during *Artemia* plus commercial diet phase (up to day 100), respectively. By July (day 430), these groups had similar testicular and undifferentiated relative areas in gonad cross-sections (approx. 50% and 25%, respectively; figs. 5.5a and 5.5c) although the ovary represented a slightly larger portion of the gonad in fish from G4, this difference was not statistically significant (fig. 5.5b). Also, no differences in the type of male germ cells (e.g. spermatogonia, spermatocytes, etc) and female germ cells (oogonia and previtellogenic oocytes) present in the gonads were noticed. By September (day 507), as the spawning season approached, testicular areas increased to nearly 90% in all fish and the sperm ducts were filled with spermatozoa and ready to emit.

V. 5 Discussion

Early exposure of sea bream larvae and juveniles to estradiol-17β did not result in precocious feminization, as all surviving fish were functional males at the end of the experiment. Despite the early accidental death of fish in some of the feeding regimes (G2, G3 and G5), which meant that analysis of the gonads when they were adults could not be carried out, it seems probable that these fish would also have developed as males since estrogen exposure during the rotifer feeding phase and *Artemia* plus initial commercial diets feeding phase (G1 and G4) did not induce sex reversal. Considering that the larval estrogen uptake was sufficient for feminization, these results suggest that sea bream larvae are insensitive to E_2 from first feeding period to 100 dph (days post hatch; the treatment time range utilised), or that any effects were lost after treatment was stopped.

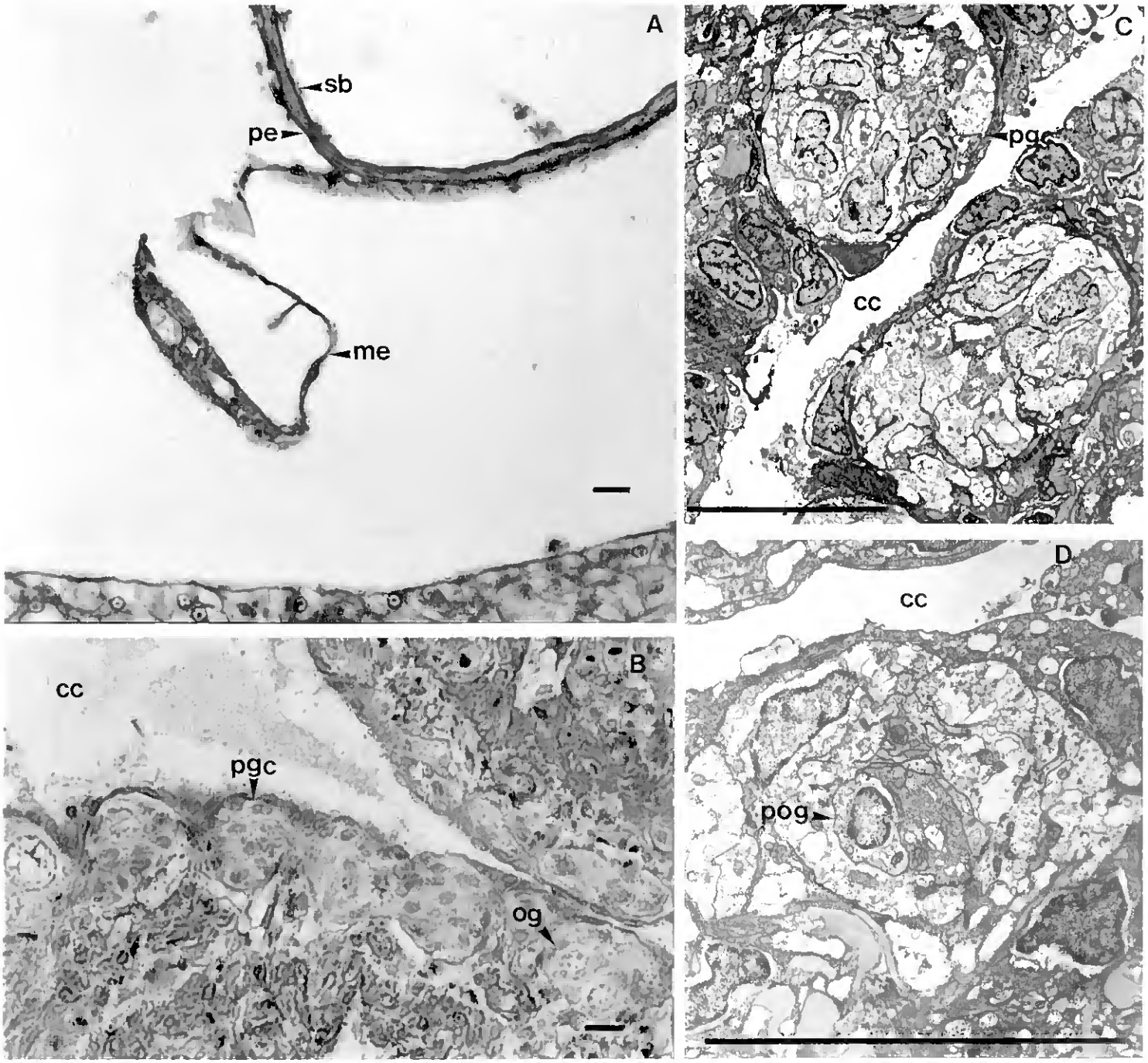


Figure 5.4 – Aspects of sea bream gonad development: A) undifferentiated gonad at early stages (87 dph); B) light micrograph of first differentiated germ cells (oogonia, og) around central cavity (cc) at 5months of age; C) electron micrographs of primary germ cell nests (pgc) and putative oogonia primordium (pOg) originated from pgc (D). Bars in photos represent 10 μm. Legend: Mesenterium (me); Peritoneum (pe); Swim bladder epithelium (sb).

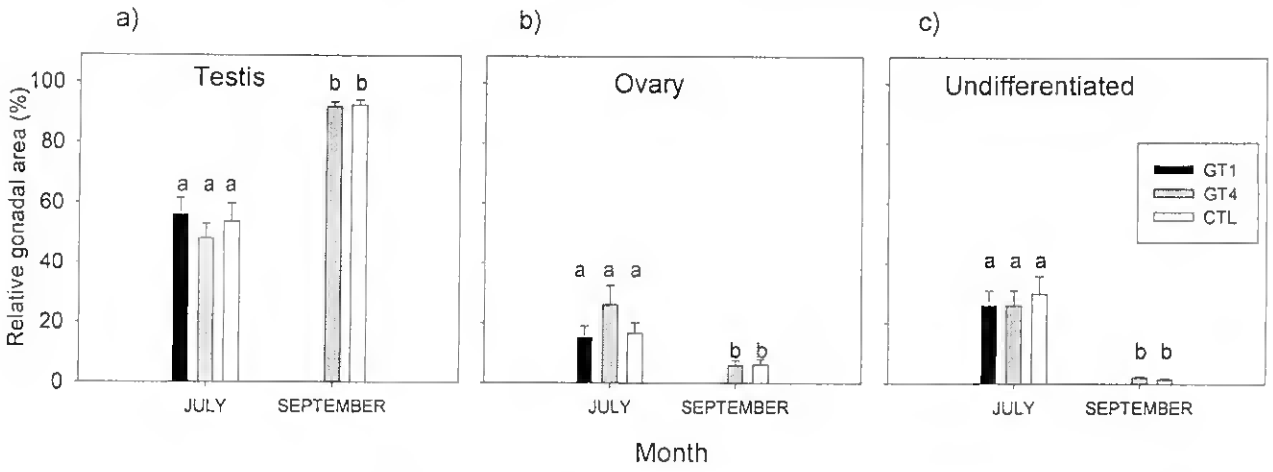


Figure 5.5 – Portion of testicular, ovarian and undifferentiated tissue in gonad cross-sections in the final sampling periods. Estrogen treated fish (G1 and G4) showed identical gonad tissue composition revealing the inefficacy of estrogen treatment in early larval stages. Different letters above bars represent statistically significant differences (two-way analysis of variance at $P<0.05$ level).

Adult sea bream can be feminized feeding daily at 3% dry feed wt/wet fish wt containing 15mg of E_2 per Kg (Condeça and Canario, 1999). Those fish were, at maximum, exposed to 90μg E_2 daily if we consider a mean experiment fish weight of 200g and that all food was consumed and the hormone taken up. This represents 0.45μg of E_2 per g of fish weight. In the present experiment, during *Artemia* 48hr EG phase, larvae were daily exposed to at least 148ng of E_2 considering the difference in larval estrogen content after the 2 first meals and fasting (early morning). Since rotifer estrogen content after enrichment was not substantially lower than in *Artemia* and considering that target biomass during larval-juvenile phase is exponentially lower than in adult fish, it is reasonable to assume that estrogen exposure was sufficient for feminization. For example, if we consider a larval weight of 0.1g this represents 1.48μg of E_2 per g of larval weight, which is 3-fold higher than in the mentioned adult feminization.

Previous studies have used estrogen enriched *Artemia* for fish feminization. This procedure achieved 100% females in both the largemouth bass (Garrett, 1989) and the lumpfish (*Cyclopterus lumpus*, Martin-Robichaud *et al.*, 1994) and was considered more effective for sex reversal than spraying hormones onto inert diets. Steroid losses into water during feeding or higher ingestion of live prey were considered as probable reasons. In the lumpfish study (Martin-Robichaud *et al.*, 1994) *Artemia* submitted to a

24hr enrichment with 10 mg E_2/l accumulated 90 ng/mg dry wt whereas the same estrogen dose in our 2-period enrichment technique achieved 1400 ng/mg dry wt. Also, in our experiment, estrogen content in enrichment water decreased sharply with incubation time, an effect not possible to explain on the basis of E_2 uptake by rotifer or *Artemia*. Several possibilities may account for this effect and include plastic adsorption from the containers wall and the lipid flocculation caused by the enrichment medium after the initial incubation hours, which could trap the steroid. Also, the very short rotifer life cycle can cause the appearance of a second rotifer generation exposed to less E_2. Despite these limitations, live prey estrogen uptake was high and the method has good perspectives for hormonal treatments in aquaculture for other fish species needing live feed during the initial developmental stages.

Teleosts exhibit a wide variety of sexual developments including ambisexuality which do not fit in with the established mammalian process of sex determination and differentiation (Reinboth, 1982; Reinboth, 1983) and also with the definition of such concepts. In mammals, sex determination is established at the time of fertilization (genetic sex) and determines gonadal sex. Once gonads have completed differentiation, the gonadal sex in turn dictates phenotypic (somatic) sex (Nagai, 1992). Such development is easier to understand in gonochoristic fish species such as, for example, the channel catfish (*Ictalarus punctatus*) which has XX-female-XY-male sex determination, although the genotype can be overridden by environmental factors, especially temperature (Patiño *et al.*, 1996). However, in hermaphrodites such as the sea bream, the genetic basis of sex is unknown and the recognition of gonadal sex is frequently very complex due to the enormous gonad plasticity (ephemeral ovaries, sex reversal, etc, Zohar *et al.*, 1978; Kadmon *et al.*, 1985; Bruslé-Sicard and Fourcault, 1997). Thus, the concepts of sex determination and differentiation are more vague. Also, very few studies on sex differentiation in hermaphroditic fish have been reported (Nakamura *et al.*, 1998). Therefore, reviews on gonadal sex differentiation either focus on gonochorists (Nakamura *et al.*, 1998) or emphasize the need of definitive criteria for the detection of the very first discrete signs of differentiation and point out their own adopted definitions (Baroiller *et al.*, 1999). In this study we distinguish between anatomical sex differentiation (gonadogenesis) and cytological sex differentiation (gametogenesis) (Martin-Robichaud *et al.*, 1994). In salmonids (major gonochorist teleost group studied for gonadal differentiation), gonads are frequently formed around

hatching time and "differentiation" takes place at the onset of exogenous feeding (about 30 days later, *Salmo gairdneri*, Lebrun *et al.*, 1982; *Oncorhynchus kisutch*, Feist *et al.*, 1990; *Oncorhynchus mykiss*, Fitzpatrick *et al.*, 1993). In the sea bream, however, gonadogenesis occurs around 90 dph and differentiation, judged by the first signs of germ cell proliferation (i.e. germ cell nests originating oogonia in the central cavity vicinity), around the age of 5-6 months (see fig.5.4). Similar differentiation criteria were adopted in another hermaphroditic fish, the protogynous wrasse, *Thalassoma bifasciatum* (Shapiro and Rasotto, 1993). Therefore, exposure to estrogen was mostly carried out before gonad development.

According to Yamamoto's theory (1969) estrogens and androgens are natural sex inducers in gonochoristic fish and hormonal treatments should start when the gonads are still undifferentiated and continued until past sexual differentiation. However, sex reversal has been achieved with exogenous hormonal treatment before sex differentiation in several species including, for example, the coho salmon, *Oncorhynchus kisutch* (Goetz *et al.*, 1979; Piferrer and Donaldson, 1989), the sea bass, *dicentrarchus labrax* (Blázquez *et al.*, 1995; Blásquez *et al.*, 1998) and the pejerrey *Odontesthes bonariensis* (Strüsmann *et al.*, 1996). Piferrer & Donaldson (1993) proposed that, at least for some species, it is not necessary to continue treatment until past sexual differentiation and that the period of maximum sensitivity for steroid administration should be tested inclusively before differentiation occurs. The same authors emphasized that effective sex reversal will depend mainly on the characteristics intimately related to ontogenesis such as number of steroid receptors present, capability to adequately metabolize steroids and fundamentally, the degree of gonadal development achieved at the time when treatment started. In the present study estrogen treatment was ineffective for the first 100 dph but, in fact, the gonadal *primordium* appeared only approximately 10 days before the end of estrogen feeding (in G4). Despite this late gonadal appearance it is not known if, during this period, other tissues as, for instance, the brain are able to metabolize steroids and can store this physiological information in a potential form or start a cascade of extragonadal effects that would be manifest later during sexual differentiation. The difference between sea bream larval estrogen content after feeding and fasting suggests that E_2 metabolism or excretion occurs. Both phenomena have been reported for fish at early developmental stages. In the steelhead trout, *Oncorhynchus mykiss,* the brain-pituitary-gonadal axis, as judge by

the presence of GtH, GnRH and several steroids (including E_2), was shown to be active at least from 48 days post fertilization (dpf, Feist and Schreck, 1996). *In vivo* metabolism of testosterone into E_2 and its glucuronide in embryos and alevins has been shown (Yeoh *et al.*, 1996b) as well as the endogenous presence of glucuronide conjugates of E_2, cortisol, 11KT, T and 17,20βP (Yeoh *et al.*, 1996a), which are water-soluble and therefore easily excreted into the environment. In the tilapia, *Oreochromis niloticus*, changes in levels of endogenous sex steroids such as androstenedione, T, 11KT and E_2 as well as *in vitro* metabolic activity was detected during, after and, surprisingly, before histological evidence of gonadal differentiation (Hines *et al.*, 1999). Furthermore, estriol was tentatively identified as metabolite of androstenedione at 29 dpf, i.e., after gonadogenesis but before ovarian or testicular differentiation. In coho salmon short immersions in E_2 of recently hatched fry (20 degree-days) were sufficient to alter sex ratios and advance the timing of sex differentiation by 70 degree-days (Foyle, 1993). Estriol, as well as E_2 and an unknown metabolite, were also identified as *in vivo* metabolites of estrone-16-C^{14} in larvae of the medaka, *Oryzias latipes* during the critical period of sex differentiation (6mm of body length, Hishida, 1965). The same author suggested that active accumulation of estrogens could be occurring in the differentiating gonad of the larvae since two thirds of the steroids remained in a free state whereas an adult fish can excrete exogenous estrogen out of the body in a few days. Considering that 60% of the estrone remained unmetabolized it is possible that this steroid could have an effective role in the 100% sex reversals achieved in all the genetic male group tested. Similarly, embryonic tissues of the Arctic charr, *Salvelinus alpinus*, incubated with E_2 as precursor converted this steroid almost exclusively to estrone and when estrone was used as precursor there was little metabolism apart from estrone conjugates (Khan *et al.*, 1997). These authors suggested, however, that the 17β-HSD isozyme present (type 2) in these tissues, with very little reductive activity, could be involved in the "inactivation" of sex steroids by converting active 17β-hydroxy forms into less potent 17-keto forms. It is possible, thus, that the high E_2 level in sea bream larvae after estrogen feeding was quickly excreted as a conjugate form or metabolized to ineffective estrogen forms, since the E_2 was almost completely cleared after an overnight fast. Steroid receptor presence during hormonal treatments is another factor of prime importance for sex reversal. Recently, two estrogen receptors, sbERα and sbERβ, have been cloned in adult sea bream tissues (Socorro *et al.*, in press). The

expression of these receptors during estrogen induced sex reversal was studied (Socorro *et al.*, 1999). Expression of sbERα (only present in testis, liver, heart and pituitary) appeared to be higher when testis predominated and was largely reduced when mostly ovarian tissue was present, whereas sbERβ had a more widespread distribution (ovary, testis, brain, liver intestine, kidney) and did not appear to be affected by estrogen treatment. It was suggested that sbERα is probably a mediator of sex change from male to female and that sbERβ is more likely to be related to a more general role in gonadal development. Furthermore, preliminary results from the same authors (Socorro, S. unpublished results) indicate that sea bream larvae from hatch to 45 dph (before gonadogenesis) seem to lack the presence of the α form of the receptor but not the β form. Therefore, it is tempting to speculate that the inexistence of sbERα during most (if not all) of the time that sea bream larvae were exposed to E_2 in the present experiment may explain the failure to observe any effects of the hormone.

The differences in results between previous studies and this study can be best explained by the fact that the ovary develops first in sea bream anyway but, contrary to what might be expected, estrogen did not "fix" gonadal sex and the gonads proceeded the normal changes after estrogen treatment was removed that lead to the development of a testis at first maturity. These results further emphasize the labile nature of the hermaphrodite gonadal sex.

In conclusion, despite the suitability of the use of live prey for hormonal treatments, sea bream larvae appear to be insensitive to estrogen exposure during the first 100 days of life. This insensibility is possibly related either to rapid conversion of E_2 into less potent estrogens and/or, most likely, with the lack of sbERα receptor.

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CHAPTER VI

CONCLUSIONS

VI.1 Steroid control of the sea bream initial stages of sex reversal

When comparing previous literature on sea bream reproductive cycle with the results from the experiments carried out in this thesis, the hypothesis that sea bream sex reversal is divided in two distinct stages is suggested by two contrasting results. First, a functional sex reversal implies the regression of the testicular portion of the gonad and the development of the previtellogenic oocytes and the growth of the ovarian portion so individuals can spawn as females. In contrast with previous reports (Zohar *et al.*, 1978), however, from estrogen-induced (chapter II) and naturally (chapter IV) sex inverting fish, it was noticed that the increase of the ovarian portion of the gonad was obtained at the expense of the testicular tissue regression and did not result in real ovarian growth. Second, the plasma E_2 levels in these fish were generally very low or below the RIA detection limit, which is in agreement with the predominance of previtellogenic oocytes in the ovary. In growing ovaries, however, during early vitellogenesis plasma E_2 levels were reported to be at their highest concentration (approx. 1700 pg. ml^{-1}, Kadmon *et al.*, 1985). Therefore, initial stages of sex reversal in sea bream are characterized by the decrease of testicular weight which allows the resting ovarian portion to be proportionally dominant within the gonad while later stages, not followed in this thesis, are characterized by the development of oocytes, real ovarian growth (vitellogenesis) and a rise in plasma E_2 levels.

Sex reversal in hermaphrodite teleosts is currently understood as complex, integrative and multi-level phenomenon (Chang *et al.*, 1997) where genetic, environmental, social and endocrine factors may interact to change the gonadal sex (Baroiller *et al.*, 1999). The high diversity of reproductive strategies in fish together with this multiplicity of sources of sex influencing factors is generally on the base of the inexistence of a common pattern of sex reversal. Sex steroids, however, if not initial triggers of sex differentiation and reversal as first suggested by Yamamoto (1969), are most likely key physiological steps in this process (Baroiller *et al.*, 1999). Estradiol-17β, in particular, has been closely associated in other protandric species with sex reversal (Kime *et al.*, 1991; Guiguen *et al.*, 1993; Chang *et al.*, 1997). Results in this thesis showed that E_2 plasma levels were low in either estrogen feminized fish (chapter II) or fish with gonads dominated by the ovary (chapter IV). However, E_2 was considered to be largely associated with feminization since it was capable of inducing

sex reversal and because estrogen treated gonads had higher capability of synthesising E_2 from androstenedione. Furthermore there was a clear positive correlation of E_2 output and ovarian tissue. However, results suggested also that exogenous estrogen effect is dependent of the current developmental status of the gonad. Experiments carried out with ethE_2 (a powerful estrogen agonist) over different sea bream populations (50 and 150g of initial total mean weight) showed that the effect of estrogen on the gonads with high proportion of undifferentiated tissue was very limited (chapter II). The same insensivity was obtained when E_2 was given to larvae and juveniles (chapter V) and it was suggested that the lack of expression of sbERα, considered as the probable estrogen receptor mediator of ovarian development in sea bream (Socorro *et al.*, 1999), in larvae until at least 45 dph was responsible for the lack of a visible effect of estrogen. Therefore, E_2 is most likely involved in sea bream sex reversal but its effectiveness depends on the degree of tissue differentiation in the gonad, namely the presence of appropriate receptors.

The initialisation of sex reversal also depends on testicular regression and this phenomenon was simultaneous with the *in vitro* decrease of 11βA and 3αA5β synthesis through the diminution of 11β-Hyd, 5β-Red, 3α-HSD and desmolase activities (chapter IV). 11βA was also present in blood plasma of two year old fish in considerable concentrations throughout the year, with higher levels when approaching the spawning season suggesting a possible role in spermiation (chapter IV). The presence of 3αA5β was not confirmed *in vivo* although 5β,3α-reduced steroids were abundant in blood plasma as verified with an antisera which detects steroids a 5β,3α configuration, including 3αA5β. Altogether these results suggest that androgens, namely 11βA, are responsible to maintain and develop the testicular tissue and when their synthesis is inhibited the testis regresses and the initial stages of sex reversal occur. The role, if any, for the strong 5β-reduction observed in the testis requires further investigation.

VI.2 Is there a female primacy pathway in sea bream sex differentiation?

Pioneering experiments carried out by Jost (1953) demonstrated that in mammals the basic embryonic program of sexual differentiation is inherently feminine and that

this "default" pathway can be overridden by the formation of the testis to produce a male. This female primacy rule is generally accepted in mammals where gonadal sex is highly stable, once it has been determined by the genetic sex, and where sex steroid hormones have essentially no effect on the gonads (Nagai, 1992). In fish, however, sexual differentiation is completely different as various types of hermaphroditism (simultaneous or sequential) at the adult stage have been described (Reinboth, 1982) and sex determination is a labile process, where steroids, environmental (mainly temperature) and social factors may redirect sex differentiation in gonochoric species or induce sex reversal in hermaphrodites (see review by Baroiller *et al.*, 1999). In particular, the existence of protandry, i.e fish that differentiate first as males and then invert to females, suggests that the mammalian rule of female primacy does not hold true in fish.

However, the situation is complex in fish and Reinboth (1988) has drawn attention to the fact that there are no protandric teleosts with a male phase (including the period until first sexual maturity) without visible elements of the female germ line in the gonads, whereas the opposite situation rarely occurs. Shapiro (1992) reinforced this view suggesting a reinterpretation of gonadal differentiation in protandric fish based in two cases: the anemonefish (*Amphiprion melanopus*) and the sea bream (*Sparus aurata*). The anemonefish live in colonies where the largest individual is the functional female, the second largest the functional male and the smaller non-breeding individuals contain both ovarian and testicular tissue in varying amounts. In a histological study in which cross-sections covering the whole gonad length of all individuals belonging to the three different groups identified Shapiro (1992) noticed that the ovarian portion of the gonad increased in parallel with the size rank. It was suggested that as individuals grow and move up in the size hierarchy within the colony they develop progressively in a female direction. When fish attained the second ranking position, the gonad, probably as a result of social cues, is redirected to develop into a testis until the dominant female disappears, when the gonad reverts to a functional ovary. In the sea bream, evidence is based on previous morphological studies (Zohar *et al.*, 1978; Zohar *et al.*, 1984). In these studies, oogonia are found to first proliferate and results in the formation of an ephemeral ovary by the age of 8 months, that subsequently degenerates resulting in a gonad that functions primarily as a testis (Zohar *et al.*, 1978, see also chapter I). Such observations reinforce the idea that in hermaphrodites the female primacy also exists.

According to Shapiro (1992) male development of these species (belonging to two different families) occupies discrete time periods in their life cycles that are probably switched on and off by a masculinizing mechanism. This concept was extended to the diandric protogynous wrasse *Thalassoma bifasciatum* (Shapiro and Rasotto, 1993). Morphologic evidence supported the hypothesis that the gonads of either primary or secondary males passed through an initial female phase prior to differentiating into an ovary or testis. The main difference found between primary and secondary testicular development was the timing of onset of the masculinizing process and the pre-existing gonadal morphology from which the testes emerge.

The present work provides further evidence supporting the hypothesis that the mammalian female primacy rule can prevail in fish as well, and makes sense not only from the morphological data but also from a physiological point of view. When feminization of sea bream was estrogen-induced, the main morphological effect was the inhibition of testicular growth and of male germ cell development beyond the spermatogonia stage, including mitosis (see chapter II). As mentioned above, it was noticed that ovarian growth was not enhanced and the increase in the proportion of ovarian tissue was made at the expense of regression of testicular tissue as the comparison of the ovarian and testicular indices clearly show. In the study of the two year old fish, gonadal development was followed, although not to the final stages of sex reversal but a similar pattern was noticed, i.e. after spawning, the increase in the ovarian tissue proportion was mainly due to the testicular regression (see chapter IV). Furthermore, the reorganization of the gonad was essentially marked by signs of testicular degeneration as the replacement of male germ cells by masses of undifferentiated tissue with yellow-brown appearance, the spreading connective tissue or the lobular disorganization rather than by evidence of ovarian growth (except the evolution of oocytes to the perinucleolar stage). These results taken together suggest the existence of a masculinizing mechanism that when switched on results in development the testis "around" a pre-existing ovary leading the fish to spawn as males and when switched off, do not allow the maintenance of the testis condition, which, at least for the initial stages of sex reversal, seems to be the responsible factor for the ovarian prevalence of the gonad.

The most impressive result that corroborates this hypothesis, however, is the remarkable parallelism of the gonad steroidogenic potential with the gonad morphological changes. The experiments with tritiated 17P on the steroidogenic potential of the gonads during estrogen-induced sex reversal showed that the difference between control and experimental groups was generally quantitative rather than qualitative. In particular, estrogen treatment caused a marked reduction in 5β-reduction, 3α-reduction, side-chain cleavage and 11β-hydroxylation (see chapter III). Initially, it was the diminution in 11β-H that lead to low yields of 11KT and especially of 11βA, whereas latter in the treatment, the simultaneous diminution of 5β-R, 3α-HSD and desmolase caused low yields of 3αA5β through the inhibition of the 17P→17P5β→3α17P5β pathway. These results further support that the inhibition of the testis development is accompanied by a "switching off" of particular enzymes rather than by the stimulation or the appearance of novel enzymes. The reduction in the activity of the above mentioned enzymes caused low yields of several androgens and this appeared to be a time-dependent process. Therefore these enzymes, and possibly these androgens, were suggested to be of importance for the maintenance of the testis condition during reproduction, which provides physiological evidence for the suggested existence of a masculinizing mechanism acting over a feminine default pathway in the sea bream, i.e., female primacy.

VI.3 Future perspectives

The inexistence of sexual dimorphism in sea bream, as in many other species, is a serious barrier to the studies of sexual inversion. Experimental designs always have to take into consideration that the sexual gonadal status of fish requires their death for histogical analyses, which is a handicap for continuous individual monitoring. Despite the development of ultrasonography as a non-invasive sexing technique (Mattson, 1991; Karlson and Holm, 1994) small, undifferentiated or ambisexual gonads are still very difficult to diagnose this way. Therefore a molecular sex marker would be most helpful for these studies. One possibility lies on the use of steroids with clear bimodal plasma content that would allow sexing through blood samples. More unrecovered radioactive precursor was associated with estrogen feminized fish (see chapter III) suggesting that

this may be a fruitful area for investigations leading to the identification of a molecular sex marker. In this respect, 11βA should also be investigated (see chapter III and IV). Although involving the death of individuals, the genetic expression of enzymes associated with the synthesis of sex steroids could also provide a potential future pathway for identification of sex markers and has the advantage of allowing the use of larvae. Aromatase (chapter II) and 11β-hydroxylase (chapter III) could be promising in this regard. Finally, further investigations to identify the *in vivo* 5β,3α-reduced steroids would be helpful to clarify sea bream seasonal steroid profile.

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