UNIVERSIDADE DO ALGARVE

Faculdade de Ciências do Mar e do Ambiente

Effects of the Goitrogenic Compounds Methimazole, Thiourea and Propylthiouracil on the Fish Thyroid Axis, *in vivo*: The Sea Bream (*Sparus auratus*) Model

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Master's degree in Marine Biology and Biotechnology

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The contents of this thesis are the exclusive responsibility of the author

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RESUMO

Goitrogens são compostos naturais ou sintéticos que podem afectar o funcionamento da tiróide e inibir a produção das hormonas da tiróide. Methimazole (MMI), propylthiouracil (PTU) e thiourea, são utilizados no tratamento clínico do hipertiroidismo em humanos e mecanismo de acção destes compostos em mamíferos é relativamente bem conhecido. Em peixes estes compostos têm sido utilizados com o objectivo de induzir hipotiroidismo, assumindo que o sistema da tiróide trabalha da mesma forma. No entanto a acção destes compostos em peixes não tem sido suficientemente estudada e os efeitos observados muitas vezes diferem com os efeitos observados em mamíferos. Neste contexto, o objectivo do presente estudo foi investigar o efeito de compostos goitrogénicos em diferentes componentes do eixo da tiróide em sea bream, um peixe teleosteo, e analisar se estes compostos podem bloquear a produção das hormonas da tiróide. Juvenis (~50 g BW) foram tratados durante 21 dias com MMI, PTU e thiourea (1mg/kg dia – através da alimentação). Os níveis das hormonas da tiróide (T3 e T4) no plasma foram medidos por radioimunoensaio, as alterações no tecido da tiróide foram observadas e o nível de expressão de um grupo de genes envolvido na produção das hormonas da tiróide (TR,TSH,TTR e Deiodinases) foi quantificado por qPCR na pituitária, fígado e rim. Unicamente MMI diminuiu a produção das hormonas da tiróide significativamente ($p<0,05$), principalmente T4. O aspecto histológico do tecido da tiróide neste grupo foi indicativo de um aumento na actividade dos folículos, tendo sido drasticamente diferente dos do grupo controlo ou do grupo tratado com PTU ou thiourea. O tratamento com PTU não bloqueou a produção das hormonas, no entanto também desenvolveu tirócitos hiperplásticos e hipertrofiados, no entanto em menor intensidade do que MMI. A expressão relativa de DI e DII no tratamento com MMI foi aumentada no fígado ao mesmo tempo que o hipotiroidismo foi induzido, no entanto a expressão de DI no rim não foi alterada. MMI também diminuiu a expressão do receptor beta das hormonas da tiróide em paralelo à diminuição dos níveis das hormonas no plasma, mas não foram observadas diferenças significativas na concentração de TTR no plasma ou a sua expressão no fígado ($p<0,05$). Os resultados obtidos demonstram que em sea bream MMI parece actuar da mesma forma do que nos mamíferos, provavelmente pela inibição da capacidade das thyoperoxidases de activar o iodo a transferi-lo a tiroglobulína. A alteração na expressão das deiodinases esta de acordo com a ideia de que sea bream em comum com outros teleosteos o principal mecanismo de controlo da função da tiróide é a deiodinaçao periferal, independentemente do eixo hipotalamo-pituitaria-tiroide.

Palavras chave: *goitrogenios, hipotiroidismo, teleosteo, folículos da tiróide, deiodinase, TRβ.*

ABSTRACT

Goitrogens are natural or synthetic compounds that suppress the function of the thyroid gland and inhibit the production of thyroid hormones (TH). Methimazole (MMI), propylthiouracil (PTU) and thiourea are used in the clinical treatment of human hyperthyroidism. Their mechanism of action in mammals is relatively well explained and such compounds have been employed in previous studies with fish to induce hypothyroidism, based upon the assumption that they work in the same way. However the action of such compounds in fish is not well studied and the effects are not always consistent with those obtained in mammals. In this context the main objective of the present study was to investigate the effects of goitrogenic compounds in the teleost fish, sea bream and analyze whether these compounds can depress TH production and determine how they influence different components of the thyroid axis in fish. Juvenile fish (~50 g BW) were treated for a period of 21 days with the goitrogens, MMI, PTU and thiourea (1mg/kg day through the food). Assessment of the impact of goitrogens on the central control of the thyroid cascade was assessed by histological investigation of thyroid follicles and thyroid stimulant hormone gene expression in the pituitary by qPCR, in addition to measurements of plasma thyroxine (T4) levels by radioimmunoassay. The effect of goitrogens on peripheral control was assessed by thyronine (T3) measurements in plasma and by determining deiodinase gene expression in the pituitary, liver and kidney. TH transport was also analysed by measuring transthyretin (TTR) plasma levels by Western blot and gene expression in liver with a specific quantitative PCR reaction. Only MMI significantly depressed TH production ($p<0.05$), especially T4. Histological appearance of the thyroid tissue in this group was indicative of increased thyroid follicle activity and was substantially different from control fish or those treated with PTU or thiourea. In MMI treated fish most of the thyroid follicles were collapsed and contained little colloid which was full of vacuoles and intracellular colloid droplets were evident in the thyrocytes. PTU treatment did not disrupt TH production, however also induced hyperplasia and hypertrophy of the thyrocytes, although it was less marked than that seen in MMI treatment. The relative expression of DI and DII in MMI treated fish was increased in the liver in parallel with the induced hypothyroidism. In contrast to the situation in the liver DI in the kidney is unresponsive to changes in the thyroid status in sea bream. MMI also decreased expression of the thyroid hormone receptor beta (TRβ) in the pituitary parallel to TH depletion, but no significant differences were found in the concentration of plasma TTR or hepatic gene expression ($p> 0.05$). These data demonstrate that in sea bream MMI seems to function in the same manner as reported in mammals, probably by inhibiting thyoperoxidases ability to activate iodine and transfer it to thyroglobulin. The alterations in deiodinase expression fits well with the general notion that in the sea bream in common with other teleosts peripheral deiodination is the primary control mechanism of thyroid function, independent of the hypothalamus-pituitary-thyroid axis.

Keywords: *Goitrogens, hypothyroidism, teleost, thyroid follicles, deidinase, TRβ.*

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LIST OF ABREVIATIONS

18S	18S ribosomal RNA
ALB	Albumin
ANOVA	Analysis of variance
bp	Base pairs
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary DNA
DBD	DNA-binding domain
DEPC	Diethylpyrocarbonate
DIT	Diiodotyrosine
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleotide triphosphate
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
HSA	Human serum albumin
HPLC	High performance liquid chromatography
IPTG	Isopropyl-beta-D-thiogalactopyranoside
IRD	Inner-ring deiodination
Kb	Kilo bases
kDa	Kilo Dalton
MIT	Monoiodeotyrosine
MMI	Methimazole
MMLV-RT	Mouse Moloney murine leukemia virus reverse transcriptase
mRNA	Messenger ribonucleic acid
MW	Molecular weight
ORD	Outer-ring deiodination
PCR	Polymerase chain reaction
Poly(A)	Polyadenylated RNA (mRNA)
qPCR	Real Time quantitative PCR
PTU	Propilthyouracil
RIA	Radioimmunoassay
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Rotations per minute
RT-PCR	Reverse transcriptase-polymerase chain reaction
sbTTR	Sea bream TTR
SDS-PAGE	Sodium dodecyl sulphate - polyacrylamide gel electrophoresis
SE	Standard error
T_3	Triiodothyronine
T_4	Thyroxine
TBG	Thyroxine-binding globulin
TG	Thyroglobulin
TH	Thyroid hormones

THBP	Thyroid hormone binding protein
TPO	Thyroid peroxidas
TR	Thyroid hormone receptors
TRE	Thyroid hormone response elements
TRH	Thyrotropin-releasing-hormone
TSH	Thyrotropin-stimulating-hormone
TTR	Transthyretin
U	Units

Thesis context

In the mean time that the principal study that gives name to this thesis was written, another work was developed aiming to update the annotation of the sequenced sea bream E_2-treated testis subtractive library clones from a previous study (Pinto et al., 2006). Annotation of clones was ensured by sequence comparison with the annotated EST libraries from sea bream and sea (MGE-Expressed-sequence-taq (EST) Libraries) or by identity searches with updated public databases. The results of this analysis are described in appendix -1. However, this thesis will focus principally in the effect of goitrogenic compounds in the sea bream thyroid axis.

The components of the thyroid axis appear to be the same in most vertebrates, and consist of a stimulating factor in the pituitary gland, thyroid stimulating hormone (TSH), thyroid hormones (T4 and T3), thyroid hormone-binding proteins (THBP), deiodinases and nuclear receptors. However, detailed information about the regulation and function of the components of the axis is still lacking in detail in fish (Power et al., 2001). Before the 1960s fish endocrinology was centered on histophysiological studies and the study of hormone physiology and their chemical nature were relatively rare, largely as a consequence of the lack of methods with which to study such aspects. Later, technological advances applied to endocrinology, like the availability of pure hormone, production of antibodies and specific highly sensitive radioimmunoassay for both T3 and T4 led to important advances in understanding thyroid hormones secretion and metabolism (Power et al., 2001). The induction of hypothyroidism has been employed as a tool in studies focusing on the role of factors involved in the thyroid axis in fish, from thyroid hormones synthesis to thyroid hormones action. In the 1990s, the emergence of molecular technologies applied to endocrine studies changed perceptions about hormone and receptor structures, hormone-receptor interactions and signal transduction, and resulted in the case of the thyroid hormone axis, in the identification and characterization of thyroid hormone receptors (TRs) and deiodinase enzymes in a

number of teleost fish species (Eales et al., 1993a; Bates et al., 1999; Mol et al., 1999). The effects of hormones are suggested to be proportional to their concentration in the circulation (Dorit et al., 1991), and an imbalance leads to endocrine disorders as a result of hypo or hyperthyroidism.

This thesis gives an overview of the thyroid hormone axis in fish, focused on biochemical, morphological and molecular levels. The effect of antithyroid compounds used to treat human diseases and hormonal dysfunction were tested on the fish thyroid axis *in vivo* using the teleost, Sea bream (*Sparus auratus*) as the experimental model. Anti-thyroid compounds have been used in previous studies with fish to induce hypothyroidism, and this is based upon theassumption that the thyroid axis works in the same way in fish as in mammals. However the results of studies of anti-thyroid agents in fish are inconclusive and not consistent with the results of similar studies in mammals. For this reason in the present study, anti-thyroid agents were administered and their effect on a range of different elements of the thyroid axis determined.

The level of thyroid hormones T3 and T4 on plasma were measured by RIA, the histological alterations in thyroid tissue were determined and the level of expression of a number of genes involved in TH production (TR, TSH, TTR and deiodinases) were quantified by quantitive real time RT- PCR (qPCR) in pituitary, liver and kidney. The present introduction will describes the conventional view of the thyroid axis, derived from mammals, and will then focus fish thyroid axis *in vivo* on the state-of-the-Art in teleosts, and finally overview what is known about the effect of anti-thyroid compounds like Thiourea, Methimazole (MMI) and Propylthiouracil (PTU).

I. INTRODUCTION

1. *Endocrine system*

As animals became larger and more complex a communication network evolved to allow cells in distant parts of the body to communicate. This network consists of the nervous system and the endocrine system which produces a variety of chemical messengers able to modulate long-term processes, which simultaneously affect many parts of the body (Dorit et al., 1991). Such chemical messengers have been grouped together and classified as hormones and are characterized by the fact that they are synthesized by specialized tissues or endocrine glands localized in specific site of the body. Endocrinology is the study of mechanisms of communication by hormones and their function.

Unlike exocrine glands, endocrine glands secrete their product directly into the blood stream, other body fluids or by diffusion between cells, rather than through a duct. The latter hormones can be produced in other tissues not specialized for hormone production, and may act in a distant organ in the body (endocrine action), locally in the same cell type which produced it (autocrine) or in neighbouring cells of a different type (paracrine) (Bentley, 1998). Hormones have traditionally been classified into four structural categories: amines, prostaglandins, steroid hormones or peptides and proteins hormones (Randall et al., 1997), or classified as: lipid soluble and lipid insoluble. Recently, a number of novel endocrine factors have been identified, such as, nitrous oxide which do not fit into the conventional classification. Hormones are synthesized by the cell and stored in membrane-bound vesicles or granules, which are released by exocytose under appropriate stimulation. Lipid insoluble hormones are secreted directly into aqueous body fluids while lipid soluble hormones generally bind to carrier proteins (Dorit et al., 1991). Secretory activities of most endocrine tissues are subject to both positive and negative feedback control and in this way the hormone regulates its own production and secretion.

The specific effects of hormones on their target tissue and their primary intracellular mode of action are achieved by their interaction with receptor proteins located in the plasma membrane or inside the cell or in the nucleus (Bentley, 1998). Lipid-insoluble hormones tend to bind to cell-surface receptors, triggering an intracellular pathway that may involve a second messenger, which in turn combines with another molecule to produce an active complex, while lipid-soluble hormones mainly combine with intracellular receptor proteins forming active complexes that modulate gene expression. The thyroid hormones, the

subject of the present thesis, are lipophilic and tend to circulate bound to proteins (THBP) and bring about their action by binding to nuclear receptors (TRs) (Yamano, 2005).

2. *Thyroid hormones – thyroxine (T4) and triiodothyronine (T3)*

Thyroid hormones (TH) are iodinated thyronines (Fig.1), present in all vertebrates (McNabb, 1992) which alone, or through interaction with other hormones and growth factors can influence intermediary metabolism, and also growth and development [7, 8]. Those hormones affect virtually every metabolic activity in the body, including the concentration and functionality of numerous enzymes, all aspects of the metabolism of fats, carbohydrates, protein, vitamins, secretion and breakdown of other hormones, as well as the response of tissues to other hormones. In higher vertebrates, THs specifically regulate basal metabolism (Zhang and Lazar, 2000; Yamano, 2005) and are key controllers of obligatory heat production in homeothermic vertebrates (Dorit et al., 1991). However, the function of THs in ectothermic vertebrates its not fully understood. In ectothermic vertebrates the best known feature of these hormone is probably their role as a metamorphosis inducing hormone in amphibians (Leloup and Buscaglia, 1977; Yamano, 2005). Information about the role of THs in fish development, mainly teleosts, has been accumulated in the last two decades. A positive correlation has been found between thyroid and reproductive status, and high levels of maternal THs has been found in eggs (Power et al., 2001). Due to these observations, THs are thought to play a key role in development and larva-juvenile transition (Power et al., 2001; Yamano, 2005).

3. *Thyroid axis*

In fish, the recognition of a tissue comparable to the thyroid gland in mammals was made in the mid/late 19th century, and the ubiquitous presence of thyroid tissue in all vertebrates became widely accepted (Leatherland, 1993). However, much of the actual knowledge concerning thyroid hormone synthesis and secretion has been derived from mammalian thyroid models. The broad steps of these models involves 1) iodine availability and absorption, 2) uptake of iodide by the thyroid tissue, 3) oxidation of iodide to transform it into active iodide, 4) thyroglobulin (TG) iodination and thyroid hormones formation, 5) storage of thyroid hormones in a TG-bound form, 6) hormone release, 7) THs transport to

target tissues and 8) cellular uptake of thyroid hormones. The control of synthesis and secretion of THs in the thyroid is regulated by stimulating hormones produced in the hypothalamus (thyrotropin releasing hormone, (TRH)) and pituitary thyroid stimulant hormone (TSH). Many factors intervene in the various steps outlined in 1-8 above, such as thyroperoxidases and deiodinating enzymes; serum transport proteins (transthyretin (TTR), thyroxine binding globulin (TBG) and albumin); and TRs.

The thyroid gland consists of functioning units called follicles, which comprise a single layer of polarized cells surrounding an internal compartment, the follicle lumen. The specialized function of these cells is to produce a high molecular weight protein called thyroglobulin (TG) and concentrate iodide within the cell. TG contains many tyrosines in its sequence which are iodinated by enzymes called thyroperoxidases (TPO). The former enzyme reacts first with hydrogen peroxide (H_2O_2), forming an oxidized enzyme that oxidizes iodide to an "active iodide" which is transferable to tyrosyl residues on TG. Additional enzyme-catalyzed reactions convert the iodinated tyrosines into monoiodotyrosine (MIT) and diiodotyrosine (DIT) and later the biologically active hormones, tetraiodothyronine (T4) and triiodothyronine (T3). The chemically modified TG molecules are extruded into the lumen and stored there as colloid. Iodide is an indispensable component and comprises 65% and 58% of the THs (T4 and T3), respectively. T4 is the predominant hormone secreted from the thyroid tissue and is converted into the biologically active form T3 by deiodination (McNabb, 1995). Over 80% of circulating T3 is produced outside the thyroid gland by peripheral conversion of T4 into T3 (http://www.thyroidmanager.org/).

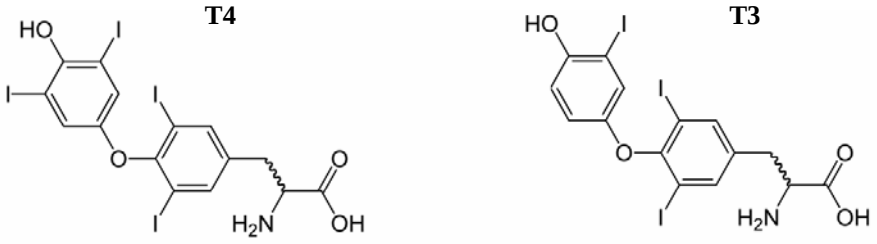


Figure. 1 - The chemical structure of thyroid hormones (TH) : 3,5,3´,5´-tetra-iodo-L-thyronine (Thyroxine, T4); 3,5,3´-tri-iodo-L-thyronine (T3)

In the general thyroid axis model, TH levels are regulated by a feedback inhibition mechanism (Fig.2) (Leatherland, 1994). In this model, the hypothalamus secrets TRH, which promotes pituitary secretions of TSH, produced by pituitary thyrotrophs. TSH in turn is necessary to stimulate the thyroid to produce and release THs. Circulatory levels of THs and TSH modulate TRH production by the hypothalamus, and the interplay of these molecules maintain circulating THs homeostasis. THs levels also influence TSH production by the pituitary and represents a further regulatory mechanism.

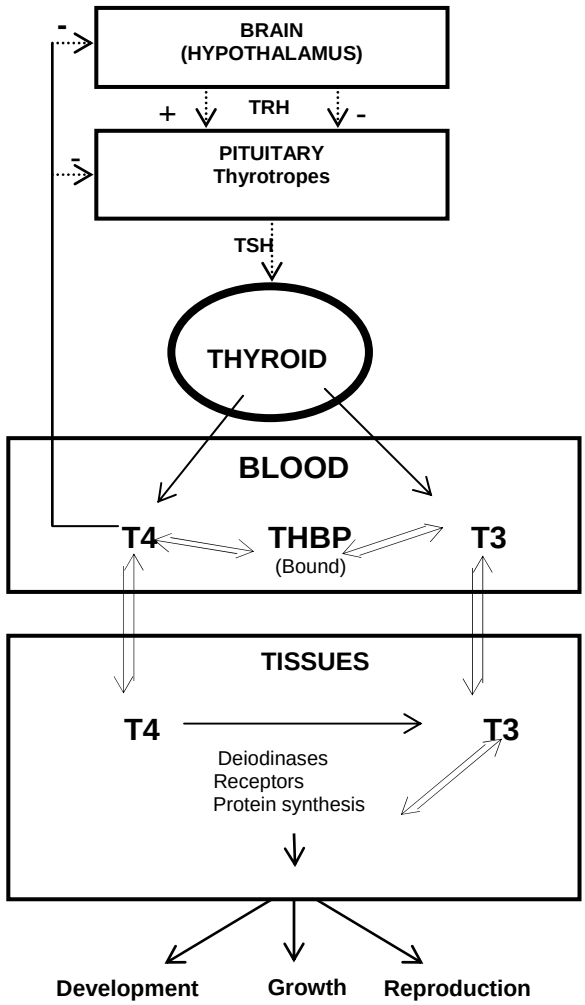


Figure 2 -General thyroid model. Thyroidal secretion of T4 and T3 is regulated by Thyroid stimulating hormone (TSH) produced by thyrotropic cells in the pituitary, controlled by positive and negative pathways from the hypothalamus. T4 and T3 in blood are mainly bound to plasma proteins (THBP), but the small free fraction exchanges tissues and can be enzymatically deiodinated by deiodinase to T3 which exchanges with blood or binds to receptors to modify specific protein synthesis and initiate biological action. T3 and T4 can also feed back to the brain and pituitary and inhibit TSH secretion. (from Eales and Brown, 1993)

Upon release into circulation nearly 99 % of T4 and T3 binds to THBP, and only a minor fraction is present in the free form in blood. Only the free hormone fraction is able to enter cells and THBP influence the rate at which the THs reach their targets and therefore their specific responses (http://www.thyroidmanager.org/). Because thyroid hormones are highly lipophilic they can easily diffuse into cells and to counteract such diffusion binding to transport proteins is essential and helps to maintain free hormone levels in balance. In larger mammals, THBP include Thyroxin-binding-globulin, transthyretin (TTR) and albumin. Besides ensuring an even distribution of THs into tissues THBP are also thought to promote their uptake at the cell membrane. Until recently it was assumed that crossing the plasma membrane of cells was a matter of simple diffusion (Robbins and Rall, 1960; Mendel, 1989). However, during the last two decades it has become apparent that THs are transported into cells by specific carrier-mediated uptake mechanisms (Divino and Schussler, 1990; Vieira et al., 1995; Hennemann et al., 2001).

After entery into cells, THs exert their action by binding to nuclear receptors that have higher affinity for T3, TRs binding induces and sometimes represses gene expression.

4. Thyroid hormone receptors

Thyroid hormone receptors (TRs) are members of the steroid-thyroid receptor super family that also includes receptors for ligands such as steroid hormones, retinoids, melatonin and vitamin D3 (Power et al., 2001). In the past few years, great progress in biochemical, functional, and structural studies has clarified the molecular mechanism of TR action. Two classes of TRs, α and β, are coded by different genes, and numerous variants exist within the two classes of receptors (Lazar, 1993). Alternative splicing of TRα or TR β generates multiple TR isoforms, and they are classified as TRα1, TRα2, and TRα3 and TRβ1 and TRβ2 (Lazar, 1993; Zhang and Lazar, 2000).

TRs function as transcription factors and directly regulate target gene expression through DNA response elements. Ligand binding triggers a conformational change in the TR, resulting in activation of the target gene (Zhang and Lazar, 2000). Although TRs can bind to thyroid response elements (TRE) on target genes as monomers or homodimers, the majority of TR is bound to TREs as a heterodimer with retinoid X receptor (Lazar, 1993).

TRs are not cytoplasmically anchored to proteins and bind to TREs even in the absence of hormone and generally repress basal transcription (Lazar, 1993).

The occupancy of nuclear receptors depends on the intracellular concentration of T3, which is influenced by factors such as cellular TH uptake and TH metabolism (Morgado et al., 2007a). The pathway for TH activation/inactivation is deiodination, a process through which T4 or T3 loses iodine units in a stepwise matter.

5. *TH activation and degradation*

In vertebrates, THs may be processed by deiodinating enzymes (deiodinases) into more active or inactive iodothyronines. Deiodinases are rare proteins as they contain a selenocysteine in their amino acid sequence and for this reason are classified as selenoproteins (Sutija and Joss, 2006). The chemical characteristics of the selenocysteine selenol group compared to a cysteine thiol group are their greater reactivity towards nucleophilic substrates and ionization at physiological pH.

Deiodinases catalyze the removal of one iodide unit from THs at the cellular level (Sutija and Joss, 2006) and as a result other less iodinated thyronines derived from T4 can be found in circulation. The removal of one iodide from the outer benzene ring of THs is referred to as outer ring deiodination (ORD) and the removal of one iodide from the inner ring is referred to as inner ring deiodination (IRD). ORD of T4 gives rise to active T3, while T4 IRD originates an inactive form of T3 (reverse T3 or rT3). T3 and rT3 can be further deiodinated to T2, T1 and T0 (thyronine). Such deiodination pathways are selectively performed by three types of deiodinases (DI, DII, and DIII) (fig. 3), which varies in substrate preference, affinity and also in tissue localization. Deiodinase DI can catalyze both inner and outer ring deiodination of T4, producing both active (T3) and inactive (rT3, T2) and inactive thyronines. DII can only deiodinate the outer ring, being the major activating hormone, in contrast DIII can only deiodinate the inner ring, being the major inactivating enzyme (Sutija and Joss, 2006).

In mammals DI is present in the liver, kidney, muscle, pituitary and thyroid gland; DII is present in the brain and lesser quantities are present in the pituitary, heart, muscle, thyroid and testis; and DIII is commonly found in the brain, skin, placenta and fetal tissue (Bates et al., 1999).

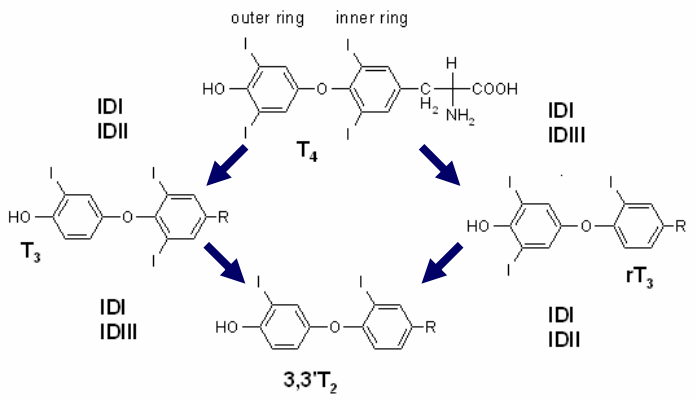


Figure 3 - Thyroid hormone deiodination pathways and Deiodinases. Deiodinase DI can catalyze both inner and outer ring deiodination of T4, producing both active (T3) and inactive (rT3, T2) and inactive thyronines. DII can only deiodinate the outer ring, being the major activating hormone. DIII can only deiodinate the inner ring, being the major inactivating enzyme (from Sutija and Joss, 2005)

6. Thyroid dysfunction

THs are essential for many basic life metabolic events and therefore adequate thyroid function is crucial for normal growth, development and healthy state. Abnormal production of THs can disturb normal THs levels (euthyroidism) leading to disease conditions like Hyperthyroidism (high THs levels) and Hypothyroidism (low THs levels). Hyperthyroidism increases the metabolic rate leading to weight loss, nervous system alterations and associated complications, etc. Hypothyroidism is associated with a lowering of metabolic rate and several associated complications (Dorit et al., 1991). In order to overcome low TH levels, the thyroid gland is stimulated by TSH to increase TH production. Associated with overstimulation of the thyroid tissue by TSH is enlargement of the thyroid gland (hyperplasia) as a consequence of increased follicular cell division and size and in extreme situations there is goiter formation (Leatherland, 1994). Hypothyroidism can be clinically treated by administration of synthetic THs. In contrast, hyperthyroidism is commonly treated by administration of synthetic antithyroid drugs, which inhibit TH synthesis. However anti-thyroid compounds can also occur naturally in some foods and exposure to them can disrupt normal thyroid function leading to goiter formation, such compounds are known as goitrogens (http://www.thyroidmanager.org/, ; Tan, 2005).

7. *Goitrogens*

Goitrogenic compounds have been divided into agents acting directly on the thyroid gland and those causing goiter by indirect action. The former group is subdivided into those inhibiting transport of iodide into the thyroid, those acting on intrathyroidal oxidation and the organic binding process of iodide and the coupling reaction of MIT and DIT, and those interfering with proteolysis, dehalogenation and hormone release. The discovery that certain drugs such as thiourea and related compounds caused hyperplasia of the thyroid when administrated to rats lead to the introduction of the thionamide series of antithyroid drugs, which are used in clinical hyperthyroidism treatment. According to their principal mode of action on iodine metabolism in the thyroid, anti-thyroid drugs are divided into two categories (http://www.thyroidmanager.org/): 1) the monovalent anions, which inhibit iodide transport into the thyroid gland, and 2) compounds that act through inhibition of thyroidal iodide binding and iodotyrosine coupling (eg. thionamides). The effect of the drugs in the first category is counteracted by exposure to excess iodide, and at times even potentiates the action of drugs in the second category. The thionamide and thiourylene drugs do not prevent transport of iodide into the thyroid gland but impair covalent binding of iodide to TG. They may be competitive substrates for thyroid iodide peroxidase, preventing the peroxidation of iodide by this enzyme (Fig4).

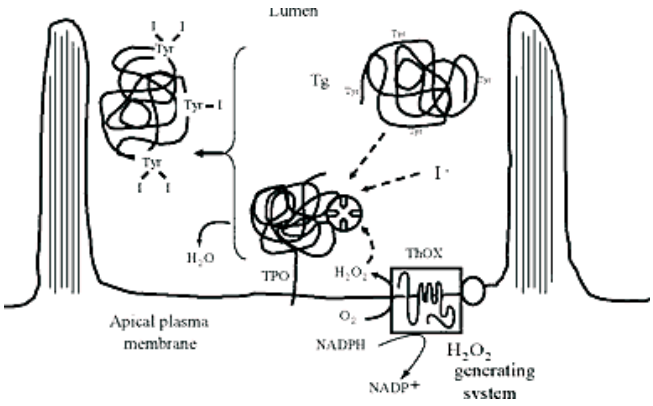
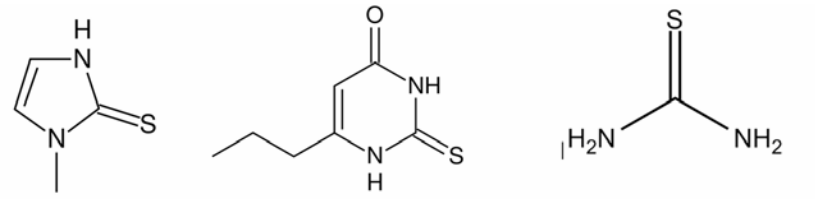


Figure 4 - TG is secreted and stored in the follicular lumen after its iodination at the apical plasma membrane-follicle lumen boundary. Via a reaction with the enzyme thyroperoxidase (TPO), iodine is covalently bound to tyrosine residues in the thyroglobulin molecule, forming monoiodotyrosine (MIT) and diiodotyrosine (DIT). (from www.thyroidmanager.org)

The thioreylene drugs (Fig 5), PTU, MMI and thiouracil, can inhibit thyroid peroxidases TPO´s ability to activate iodine and transfer it to TG (Davison et al., 1978). However these drugs act by different mechanisms. PTU interacts with the activated iodine producing a reversible inhibition of TPO. MMI interacts directly with the TPO enzyme and inhibits it irreversibly (Crane et al., 2006). PTU is a methylmercaptomidazole which reduces circulating levels of T4 and T3 and increases circulating levels of TSH. PTU is generally believed to produce deleterious effects in animals by causing a dose dependent reduction in circulating levels of thyroid hormone, caused by the ability of PTU to directly inhibit the function of the thyroperoxidases enzymes. PTU and MMI partially inhibit the peripheral deiodination of T4 and in this way its biological action (van der Ven et al., 2006). These drugs are accumulated by the thyroid and degraded at this site, since they are substrates of the peroxidases (http://www.thyroidmanager.org/).



Methimazole **Propylthiouracil** **Thiourea**

Figure 5 - Chemical structure of the Thyroperoxidases inhibitors, methimazole (MMI), Propylthiouracil (PTU) and Thiourea. A thiocarbamide group is responsible for goitrogenic activity in MMI and 6-n-propyl-2-thiouracil (Elsalini and Rohr, 2003).

A number of researchers have employed those compounds in order to disrupt the thyroid axis and investigated the metabolism and role of THs activity in fish reproduction, embryogenesis, larval development, growth etc. (Mol et al., 1999; Manzon et al., 2001; Matta et al., 2002; Crane et al., 2006; Swapna et al., 2006; van der Ven et al., 2006). However, the effectiveness as antithyroid agents and depletion of THs was not consistent. Different responses in T3 and T4 levels were observed between different species and state of development. Is believed that such compounds have a concentration-dependent effect (van der Ven et al., 2006). However, until now no standardized method had been validated to assess thyroid disruption in fish.

8. *Thyroid axis in fish*

The majority of research about the hypothalamus-pituitary-thyroid axis in fish has been focused on teleosts. Thyroid structure and function in teleosts resembles that of other vertebrates in relation to follicular organisation and primary hormones secreted (Iodine containing L-thyroxine,T4). However, unlike mammals, thyroid follicles in fish do not generally form a gland (exception is tuna fish and parrot fish) as they lack a capsule and instead they are spread throughout the connective tissue of the pharynx (Leatherland, 1993; Bentley, 1998). The organization is also variable between and within fish taxa and follicles can be dispersed in connective tissue near the pharyngeal region, located next to the ventral aorta, or can even migrate from the subpharynx and associate with the kidney (Eales and Brown, 1993). Recently in zebra fish was found evidence for hormone production not only in thyroid follicular cells, but also in an anterior non-follicular group of cells, which are able to produce THs in an independently of the pathway leading to thyroid follicle formation (Wendl et al., 2002).

In teleosts the study of the central control is rudimentary but appears to be limited to the control of production and secretion of T4 (Eales and Brown, 1993). This differs from the mammalian thyroid system which is driven principally via hypothalamus-pituitary-thyroid axis and which regulates both T4 and T3. In all vertebrates important THs activation is controlled outside the thyroid and deiodination of T4 to T3 occurs principally in peripheral tissues, principally in the liver, (but also in brain, kidney and gill) rather than in thyroid itself (Power et al., 2001). In addition, and in contrast to mammals where plasma T4 largely exceeds T3 levels, plasma T3 levels in teleosts may exceed that of T4 (Power et al., 2001).

There are two fundamental differences in Iodide metabolism between fish and mammals. Fish are able to take up its requirements of iodide from the bathing via the gill surface, even in fresh water that has less iodide than salt water. Although like mammals iodide can be taken up from the diet as well, however the supply from water is generally sufficient (Eales and Brown, 1993).

In fish the functional role of thyrotrophin release hormone (TRH) in regulating TSH is not well established, although recent experiments using pituitary cells isolated from the bighead carp indicate that TRH exposure upregulates TSH messenger RNA levels. TRH bring about

its action by binding to a G-protein coupled receptor and fish in common with mammals possess two forms (Yamano et al., 1994; Yamano and Inui, 1995).

Agnathans, chondrichthyes, and teleosts express the three isotypes of deiodinases DI, DII and DIII, which are responsible for the peripheral fine-tuning of THs bioactivity (Orozco and Valverde-R, 2005), although, tissue distribution and deiodinases activity in fishes is not identical from those of the mammalian enzymes (Sanders et al., 1999). Tilapia type I deiodinase catalyses both ORD and IRD, type II catalyses only ORD and type III catalyses only IRD (Power et al., 2001). As in higher vertebrates, in fish two TR (TRα and TRβ) genes has been cloned from several species and their functional differentiation has been suggested, specially during metamorphosis and early development (Yamano, 2005), indicating that THs function can be modulated at the receptor level, which may partly explain why TH can influence on various parts of the body simultaneously. In contrast to higher vertebrates, where has been established that TR regulates its responsive genes by binding to a cis-acting element of the gene, in fish such direct target genes had not been determined (Yamano, 2005). Relatively little is known about THBPs in fish until recently. TTR was cloned in a teleost fish "*Sparus auratus*" (Santos and Power, 1999), but before that time THs had only been found in plasma bound to albumin and lipoproteins. TTR was shown to bind THs (Santos and Power, 1999; Santos et al., 2002; Morgado et al., 2007c). Now is clear that this protein is involved in TH transport and metabolic processes related to the thyroid axis in fish (Yamauchi et al., 1999; Power et al., 2000), being suggested a direct or indirect control of TTR expression by THs levels.

9. ***Objective***

The objective of the present study was to analyse the effect of three anti-thyroid agents used in medical treatment of hypothyroidism in humans, on the thyroid axis of a teleost fish. These anti-thyroid compounds have been used in previous studies with fish to induce hypothyroidism, and this is based upon the assumption that the thyroid axis works in the same way in fish as in mammals, however, the results were inconsistent.

In order to clarify whether several goitrogens can depress serum TH concentrations and test the assumption that the thyroid axis works in the same way in fish as in mammals, the major goitrogenic compounds, MMI, thiourea and PTU were tested on the fish thyroid axis *in vivo* using the teleost, Sea bream (*Sparus auratus*) as the experimental model.

The effect of these compounds were analyzed in different elements of the thyroid axis:

- THs serum concentrations were measured by RIA, and the role of the Hypothalamus-pituitary-thyroid axis and peripheral control were evaluated by analyzing T4 levels and T4/ T3 ratio, respectively.

- The morphology of the thyroid follicles was assessed by staining of sections of sea bream thyroid tissue in order to detect eventual goiter formation and thyroid activation.

- Transthyretin and total serum proteins concentrations were measured by Western blot and Lowry assay, respectively, in order to detect eventually effects of goitrogenic compounds in THs transport proteins.

- Gene expression of TSHβ, TTR, TRα, TRβ and Deiodinases I, II and III were quantified by Real time quantitative PCR (qPCR) in order to investigate the mechanism of action of thyroid axis in fish.

Altogether, this information will help to understand if MMI, thiourea and PTU are effective anti-thyroid agents in fish and consequently if they can be efficiently used as hypothyroidism inducers in experimental conditions.

10. The experimental model – Sea bream

Sea bream, *Sparus auratus*, (Fig 6.) is a marine fish, which lives at 1-150m depth. It belongs to the Family sparidae, Order perciformes and Class actinopterygii. It is a protandric hermaphrodite species, maturing first as male (during the first or second year of age) and after the second or third year of age, as female. Spawning generally occurs in the wild from October to December, with sequential spawning during the whole period (http:// www.fishbase.org).

Due to the ready availability of sea bream in southern Europe and its importance as an aquaculture species, considerable research has been carried out with this species which is

used as a physiological model of temperate marine teleosts. A large number of resources are now available for this species, including genomic libraries, complementary DNA (cDNA) libraries, microarrays and a radiation hybrid (RH) map.

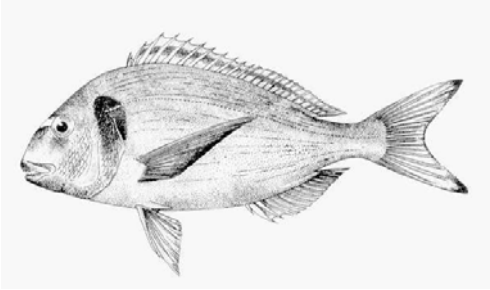


Figure 6 – The experimental model: Sea bream (*Sparus auratus)* (Taken from www.fishbase.org)

In the sea bream the thyroid follicles are visible as early as four days post-hatch when larvae are still poorly developed (Santos et al., 2002) and maternal THs has also been detected in sea bream eggs, suggesting that THs may contribute to early development (Power et al., 2000) and to growth and maturation in adult sea bream.

The application of techniques of molecular biology in sea bream has resulted in the identification and characterization of TRs and the three iodothyronine deiodinases enzymes. The TRs (TRα and TRβ) were also found in this teleost and the tissue distribution of TRβ transcripts assessed, indicating similar expression in skeletal muscle, heart, intestine, brain, kidney, liver and gill. In addition was already demonstrated, *in vitro,* that TRβ binds T3 with greater affinity than T4 (Nowell et al., 2001). The only studies concerning the tissue localization of TTR transcripts in fish have been carried out in the sea bream, being this produced in liver, intestine, brain, skin, heart, skeletal muscle, kidney, testis, gills and pituitary (Funkenstein et al., 1999; Santos et al., 2002). TTR was cloned from a liver cDNA library (Santos and Power, 1999) and was found to bind preferentially T3 (Morgado et al., 2006).

II. METHODS

1. Animal and experiment conditions

Immature sea bream (approx. 50g body weight) were obtained from TIMAR (Tavira, Portugal) and maintained in open sea water circuits at the experimental station Ramalhete Marine Station (Faro, Portugal) at normal ambient temperature (20-21 ºC) and salinity (36 parts per thousand, ppt) for October in Algarve, Portugal.

Fish were weighed and transferred to four different tanks (65L) labeled as follows: control (average wet weight fish (W)=57,86±7,38g); thiourea (W=60,29±10,27g), PTU (W=58±6,76g) and MMI (W=59,14±11,82g) at a density of seven fish per tank. Fish were acclimated to the experimental circuit for 2 weeks with a 12 h light/dark photoperiod and daybreak set at 07.00h. Water temperature and salinity were 20-21 ºC and 36 ppt, respectively during the experiment. Fish were fed twice daily during acclimation and the experiment and the total food administered per day was 1.5% of total fish weight per tank. A commercial sea bream diet (Dourasoja size 2mm) was used. Maintenance and manipulation of fish was conducted in accordance with the Guidelines of the European Union Council (86/609/EU).

2. Administration of goitrogenic compounds

All the goitrogenic compounds used to treat the fish were administered via the diet in such a way that the daily exposure would be approximately 1 µg/g wet fish weight. The cumulative concentration at the end of the experiment (21 days) in a 50g fish (the average size) was calculated to be 1mg. For the treatment of food, chemicals were dissolved in 5 ml of ethanol to the appropriate concentration and then the food pellets were sprayed with the chemical solution and fed at the desired dose to the fish. Particular care was taken to ensure food was evenly coated with the chemical solution and to reduce risks for experimenters this was carried out in a fume cupboard and the food left until all the ethanol had evaporated. In order to avoid oxidation of the goitrogenic compounds, treated food was prepared weekly with freshly prepared solutions and stored in an airtight bag at 4 ºC in the dark. Just before feeding the fish with the food its palatability was increased by spraying it with cod liver oil.

3. Tissue sampling

After 21 days of treatment fish were anaesthetized in 0.02 % (v/v) phenoxyethanol (Sigma-Aldrich), weighted, length measured and a blood sample removed from the caudal region using a heparinized syringe. Blood was centrifuged at 1000g for 10 min at 4 ºC and the plasma frozen in liquid nitrogen and stored at -20 ºC. Fish were then killed by decapitation and the kidney, pituitary and liver rapidly dissected out. The tissues were immediately snap frozen in liquid nitrogen and stored at -80 ºC until analysis. Tissue from the insertion of the gill arch into the lower mandible (see below) was fixed in Bouin-Holland for subsequent thyroid histology.

4. Histological procedure

4.1 *Fixation and processing*:

Thyroid tissue in fish is located in the lower jaw in the region of insertion of the gill arches with the pharynx. Since the thyroid tissue in fish is not encapsulated it tends to be difficult to identify and specifically extract and for this reason the entire pharyngeal region was fixed in Bouin–Holland solution (ratio 1 volume of tissue: 10 volumes of fixative). Tissue was fixed for 4 days at room temperature with occasional agitation, fixative was removed and tissue rinsed in distilled water until excess fixative was removed. Tissue was subsequently decalcified in a solution of EDTA, pH 8 (GE) at room temperature with occasional agitation over 7 days.

Paraffin blocks of the fixed tissue for sectioning were prepared by dehydration through a graded ethanol series from 70 to 100%, followed by saturated xylene (100%) and finally embedded in paraffin (Histosec, Merk, Germany) using an automated tissue processor (Leica, TP1020). Sections of embedded tissue (8 μm) were cut using a rotary microtome (Leica, RM2125). Serial sections were mounted on glass slides coated with poly-L-lysine (Sigma-Aldrich, Spain) and dried overnight in an oven at 37°C.

4.2 *Staining*:

In order to identify the thyroid follicles in the sections these were stained using Cleveland–Wolfe trichrome (Cleveland and Wolfe 1932). This method, more classically applied for identification of endocrine cells in the pituitary gland gives good differentiation of thyroid

tissue, as the colloid present in the follicle lumen stains bright red/orange and the thyrocytes of the follicle stain purple.

For staining, paraffin was removed from sections by immersion in xylene and tissue was then rehydrated by passing it through a graded series of ethanol (100% → 50%). Mercury, one of the constituents of Bouin–Holland fixative may interfere with staining and therefore was removed from tissue sections by immersion in 1% iodine in 70% ethanol, followed by immersion in 5% sodium thiosulfate and rinsing in distilled water just prior to staining. Tissue sections were stained in Erlich's haematoxylin for 5 min, "blued" in tap water and washed with distilled water. Subsequently, tissue sections were stained in 1% aqueous erythrosine for 5 min, rinsed in tap water and distilled water and then stained for 10 s in 2% Orange G (in 1% phosphotungstic acid), then rinsed in distilled water before staining with Aniline blue (0.25% aqueous, acidified to pH 3.5) for 90s. Finally, the sections were rapidly rinsed in ethanol 100%, followed by xylene, and mounted for definitive preparation in DPX. Photographs of stained sections of thyroid tissue were taken using an Olympus BH-2 optical microscope coupled to an Olympus DP11 digital camera. Image processing was carried out using open source software, Image-J (http://rsb.info.nih.gov/ij/).

5. Biochemical analysis

Biochemical analyses were developed in order to quantify total protein and total thyroid hormones (T3 and T4) in plasma. Total protein was quantified in order to establish if treatments modified the concentration of plasma protein compared to control fish and also to compare/normalize protein expression of the thyroid hormone binding protein transthyretin (TTR), which was analyzed by western blot. The plasma TH levels were measured in order to assess the effectiveness of treatment with goitrogenic compounds which should cause a significant reduction in circulating THs.

5.1 Total protein in plasma

The total protein concentration in fish plasma was determined using the Lowry Method (Lowry et al., 1951). This assay is based on the reaction of proteins with copper ion in alkaline solution. As a result, complexes are formed, which react with the Folin-phenol reagent (a mixture of phosphotungstic acid and phosphomolybdic acid in phenol), and the

product of this reaction becomes reduced to molybdenum/tungsten blue and can be detected colourimetrically by absorbance. A standard curve with know quantities of protein is used and because the absorption of light is proportional to the concentration of protein present this relationship can be used to determine the concentration of protein in samples.

The assay was performed with bovine serum albumin as the standard and the mass of protein present in each sample calculated using the linear equation applied to the standard curve. The protein was diluted in NaOH 0.1N, making a total volume of 100 µl. The concentration of protein was determined in collected fish plasma in duplicate using 5µl of a 1/10 dilution of plasma in each reaction together with 1000 µl of C solution (1ml: 0,5%$CuSO_4$ in 1% $NaCO_3$; plus 50ml: 2%Na_2CO_3 in,0.1 N NaOH). The reaction was then vortexed and tubes maintained for 10 min at room temperature and subsequently 100 µl of Folin Reagent (50%) was added and the absorbance measured immediately at 745 nm.

5.2 Western blot of transthyretin in plasma

TTR in plasma was determined by western blot analysis using the ECL system (ECL plus western blotting detection system, Amersham Biosciences). For western blot a standard curve was prepared with serial dilutions of standardised sea bream recombinant TTR (from 35 to 75 ng) and was always run in parallel to four plasma samples collected from each experimental group (Control, thiourea, MMI, PTU). TTR signal in the developed western blots was quantified by densitometry using the software Quantity One (free trial- Bio-Rad, USA). Solution and gel compositions needed for SDS-PAGE and immunobloting are presented in Appendix.

5.2.1 SDS-PAGE

Prior to electrophoresis, samples were prepared in 2x loading buffer containing dithiothreitol (DTT) and boiled for 10 min at 100ºC to ensure protein denaturing. Minigels of 1,5 mm thickness were prepared in a dual gel vertical caster system (Mighty Small™ SE245, Hoefer, San Francisco) with 10-well combs. After polymerization of polyacrylamide gels they were transferred to the electrophoresis unit (Mighty Small II SE250/260, Hoefer) and samples were loaded and run in a Tris-Glycine running buffer at constant power of 40 mA. A prestained protein ladder (#SM0671, Fermentas) was always

included in electrophoresis to give an estimate of the molecular weight of the proteins analysed.

The gel was used for immunoblotting after transferring the fractionated proteins to hydrophobic polyvinylidene difluoride (PVD) membrane (Hybond-P, Amersham Bioscicences). The transfer and immunoblot processes, were performed according to the suppliers manual and are now briefly described. On completion of SDS-PAGE, a sheet of Hybond-P membrane was cut to the size of the resolving gel and wet in 100 % methanol for 10 s for activation, washed in distilled water for 5 min and then equilibrated in transfer buffer for at least 10 min. Several sheets of Whatman paper were also cut to exactly the same size as the polyacrylamide gel and also soaked in transfer solution. Immediately after polyacrylamide gel was ran it was also soaked in protein transfer buffer for 10-20 min. After equilibration a transfer "sandwich" was assembled by placing the Hybond-P membrane in close contact with the gel and then placing 6 sheets of absorbent Whatman paper on each side. The "sandwich" was fastened into an electroblotting cassette and placed inside a vertical electroblotting tank (TE22 Mighty small tank transfer, Hoeffer) filled with transfer buffer. After transfer, carried out for 1 h at 300mA with water cooling, the Hybond-P membrane containing the transferred proteins was removed from the cassette, rinsed in 1xPBS, wet in 100% methanol and washed for 5 min in distilled water before incubation in skimmed milk blocking solution overnight at 4 ºC.

5.2.2 Immunoblotting and detection by chemiluminescence

Detection by chemiluminescence was performed using the kit ECL Plus Western Blotting Detection System (Amersham Biosciences). Basic steps in the protocol were done according to the supplier's instructions and after optimization the procedure was the following:

The blocked membrane was rinsed in 1x PBS with two changes to remove excess blocking solution and incubated with rabbit anti-seabream TTR sera, produzed in rabbit against purified recombinant sea bream (Morgado et al., 2006) (optimized dilution of 1:10 000) for 1 h at room temperature. Excess antisera was removed by washing membrane with excess (4 ml/cm^2) PBS/Tween 20 washing solution followed by another 3 x 5 min washes prior to

addition of the secondary antibody ECL anti-rabbit IgG horseradish peroxidase linked, from donkey (1:100 000 dilution, Amersham Biosciences). Membranes were incubated with the secondary antisera at room temperature for 1 h and rinsed twice in washing solution prior to a 15 min wash in excess (4 ml/cm^2) wash buffer followed by another 3 x 5 min washes. For detection reagents A and B supplied in the kit, previously equilibrated at room temperature for some minutes, were mixed with the proportion 40:1 to allow a final volume of 0.1 ml/cm^2 of membrane. The membrane was placed in as sheet of cling film (after removing excess wash buffer) with protein side up. The mix detection reagent was evenly distributed on the membrane surface and incubated at room temperature for 5 min. Excess reagent was carefully drained by holding membrane corner against an absorbent tissue. The membrane was then placed inside a transparent plastic sheet, removing any air bubbles and transferred to an X-ray film cassette protein side up. In the dark room an autoradiography film (Hyperfilm ECL, amersham biosciences) was placed on top of the membrane, exposed for 15 s. The film was developed (using Kodac fixer and developer from Sigma-Aldrich) and based on its appearance exposed for more time if needed (estimated time could usually go from 1 min to 1 h) until the desired band intensity and clean background was achieved.

5.3 Radioimnunoassay for T4 and T3

5.3.1 Overview of a radioimmunoassay (RIA)

The basic principal of all immunoassays is the quantification of antigens (peptide and proteins) through their specific interaction with antibodies (Chard, 1990). In RIA, the quantification of an antigen depends on the progressive saturation of specific antibody molecules and the equilibrium which is established between the proportion of antigen which is free or bound to the antibodies. The distinction between the free and bound fraction of the antigen is made by incorporation of a radioisotope that works as an antigen "tracer" and permits the proportion of antigen bound or free to be estimated. The most frequently utilized radioisotopes utilized are tritium (^{3}H) or iodine 125 (^{125}I). In the present study RIA was used to quantify the concentration of THs (antigen) in fish plasma, using radioactively labeled hormones ([^{125}I]-T4 and [^{125}I]-T3) as tracers and antiT4 or antiT3 antibodies.

The TH RIA is based on the competition between radioactively labeled T3 or T4 and molecules of free T3 and T4 present in sample for a constant concentration of antibody (antiT4 or antiT3 sera) molecules. Initially, only the labeled hormone is bound to the antibody but when free hormone is added both compete for the same antibody binding sites. This means that the higher the concentration of free hormone the higher the proportion bound to the antibody and the greater the proportion of labeled hormone which is displaced. Before carrying out the RIA, an antibody dilution curve is performed trough serial dilutions of the antibody, which are incubated with a fixed amount of labeled hormone. The proper amount of antibody chosen is that which will bind 50% of the labeled hormone (Fig 7A). At this concentration it is apparent that the addition of further ligand must lead to a substantially greater increase in the free fraction than the bound fraction. In contrast, if a much higher concentration of binder is chosen, the amount of ligand required to produce a significant shift in the bound and free fractions will be much greater.

In order to establish the relationship between radioactive measurements (counts per minute) and the concentration of free hormone added, a standar curve is designed (Fig 7B). This curve involves the incubation of fixed amounts of labeled hormone and antibody with known concentrations of free hormone. The percentage of the labeled hormone bound to antibody is progressively reduced with increasing concentrations of standard (unlabeled hormone).

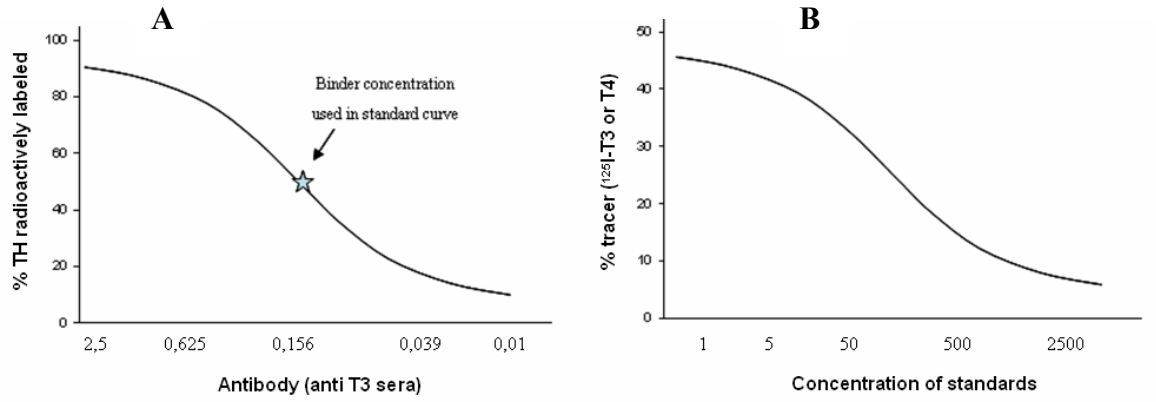


Figure. 7- (A) Antibody dilution curve: Serial dilutions of the antibody are incubated with a fixed amount of tracer ligand. The amount of antibody chosen is that which will bind 50% of the tracer. (B) Standard curve: fixed amounts of tracer ligand and binder are incubated with varying concentrations of standard unlabelled ligand. The percentage of the tracer bound to antibody is progressively reduced with increasing concentrations of standard (adapted from Chart, 1990)

In order to calculate the final concentration of hormone in the sample, some characteristics need to be measured. 1) Maximum ligation of the assay (M), which is achieved by adding labeled hormone in excess to the specific antibodies in the absence of competition (in absence of free hormone all the binding sites of the antibody will be coupled by the radioactive hormone); 2) total counts (T), or total quantity of labeled hormone, added to the assay and this is determined for duplicate tubes which contain only radioactive "tracer" and 3) none-specific binding, that is the radioactive hormone which will reside in reaction tubes in the absence of specific separation as a consequence of none specific sticking of the radioactive "tracer" to the tube and other components of the assay.

In order to quantify the labeled antigen which is specifically bound to antibody the bound and free fraction of antigen need to be separated. Separation can be carried out using a number of different methods and the success of the method depends on the nature of the antigen. Two of the principal methods involve either i) the use of activated carbon or ii) a secondary antibody. In the first mechanism, activated carbon is added to the reaction tube and free molecules in solution enter the porous charcoal while antibody bound antigen complexes are too large. The charcoal containing unbound antigen is separated by centrifugation and the antigen bound to antibody remains in solution and can be counted in a gamma or beta-radiation counter depending on the radioactive "tracer" utilized. In the second method, the separation can be ensured using a secondary antibody directed against the primary antibody specific for the antigen being measured, in this case the antigen-antibody-secondary/antibody complex precipitates due to changes in solubility and the amount of radioactive "tracer" bound in the antibody complex can be measured in a gamma counter.

5.3.2 T3 and T4 RIA conditions

Thyroid hormone plasma concentrations were measured using specific and reproducible radioimmunoassay (RIA) for T3 and for T4. Briefly, plasma samples were diluted (1/10) in phosphate buffer (0.01M pH 7.60), heat extracted (1h 30 min at 70ºC) and then cooled on ice and centrifuged for 5 min, at 4ºC and 3000 rpm. The total assay volume was 300µl and was composed of 100µl of standard or diluted plasma (15 µl of 1/10 diluted plasma in 85 µl

buffer), 100 µl of anti-T4 sera (1:8000, ~3% cross-reactivity with T3; T2652 - Sigma-Aldrich) or anti-T3 sera (1:15000, <0.01% cross-reactivity with T4; T2777 - Sigma-Aldrich) and 100 µl of tracer ([^{125}I]-T4 and [^{125}I]-T3, respectively). Assays were incubated overnight at 4ºC and separated by adding a secondary antibody (100 µl; diluted 1:10 in 0.01% (w/v) rabbit IgG; Sigma- Aldrich) to all tubes with the exception of those used to determine total counts. Tubes were mixed and incubated for a further 20 min at room temperature, after which 300 µl of an ice cold PEG solution (7.5 % w/v PEG 8000; Merck) in assay buffer was added, and the tubes were centrifuged for 20 min, 1500g at 4ºC. The supernatants were decanted, and the pellets were counted in a gamma counter (Wizard, Pharmacia-LKB).

6. Molecular analyses

6.1 RNA extraction

Total RNA was extracted from the whole pituitaries and 25-100 mg of kidneys and liver sections using TRI reagent (Sigma-Aldrich) according to the protocol and recommendations provided by the manufacturer. Extracted RNA was resuspended in 150-250 µl (kidney), 100-300 µl (liver) and 10 µl (pituitary) of DEPC-treated water. Total RNA was quantified using a commercial kit (Quant-iT RNA Assay Kit and Qubit fluorometer, (Invitrogen, Carlsbad, CA. USA), from 1 µl of RNA dilutions (1:10 - pituitary, 1:25 - Kidney and 1:50 - liver). Quality of extracted RNA was assessed by measuring absorbance at 260 and 280 nm (1Abs260=40µg/ml of RNA; ratio Abs260/Abs280 estimates RNA purity and should be higher than 1.6).

To eliminate possible contaminating genomic DNA prior to cDNA synthesis, 1µg of RNA was treated with DNase using the DNA-free Kit (Ambion, UK), in a 20 µl reaction volume, and following the suppliers instructions. At the end of treatment, 12 µl of DNA-free RNA was recovered.

6.2 Complementary DNA (cDNA) synthesis

Synthesis of cDNA was carried out in a 20 µl final reaction volume using 500 ng of each DNase-treated RNA, 1 mM of dNTP mixture, 50 ng of random hexamer primers (pd(N)6, Amersham Biosciences) and sterile water (Sigma-Aldrich). The reaction was heated at 65

ºC for 10 min to heat-denature RNA and incubated on ice for 10 min before adding the final reaction components: 25 U of RNAguard RNase inhibitor (Amersham Biosciences), 40 U of MMLV RT (reverse transcriptase) and 5x RT buffer (Promega). The cDNA synthesis reaction was incubated for 10 min at 25 ºC followed by 50 min at 42 ºC and terminated by incubation for 10 min at 72 ºC.

Before starting the gene expression analysis, 18S cDNA amplification by RT-PCR was used as an indicator of sample concentration and quality. This gene is usually used as an internal control in semi-quantitative or quantitative relative RT-PCR, to normalize for the input RNA amounts and RT efficiencies between different cDNA samples, since it is expressed in relatively constant and high levels in all types of cells, or simply to estimate the cDNA quality.

6.3 Polymerase chain reaction (PCR)

6.3.1 Overview

The polymerase chain reaction its one of the most frequently used tool in molecular biology, since it allows amplification of gene targets, which permits detection of transcripts present in tissue at low abundance or in tissue samples of very small size. It uses the ability of a thermo-stable DNA polymerase enzyme (Taq polymerase) to extend short single stranded synthetic oligonucleotides "primers" during repeated cycles of heat denaturation, primer annealing and primer extension. The primers are designed to bind the DNA fragment to be amplified. The Taq polymerase uses the target DNA added to the reaction as a template for primer extension. At each cycle, more DNA is synthesized, providing additional template. The reaction proceeds in an exponential manner, doubling the amount of target in each cycle, until one of the reagents becomes limiting and the reaction reaches a plateau, or until the Taq polymerase has lost its activity.

6.3.2 Amplification of the internal control 18S to evaluate cDNA sample concentration and quality

The PCR for 18S ribosomal RNA (18S rRNA) contained 0.5 µl of each cDNA (approximately 12.5 ng), 10 pmol of forward and reverse primer, 500 µM dNTPs, 1.5 mM $MgCl_2$, 0.5U of *Taq* DNA polymerase (Promega, VWR, Portugal), 1x PCR buffer and

molecular biology grade water (Sigma-Aldrich) to a final volume of 10 µl. Thermocycling conditions consisted of an initial denaturing step of 95 ºC for 1 min, followed by 18 cycles of 95 ºC for 30 s (denaturing), 59 ºC for 30 s (annealing) and 72 ºC for 30 s (usually 60 s/Kb expected, extension). A final elongation step was performed at 72 ºC for 1 min and PCR products were analyzed by agarose gel (1 %) electrophoresis (solution and gel compositions is described in Appendix).

Typically, the 18S assay yields visible amounts of PCR products (analyzed by agarose gel electrophoresis) at less than 22 cycles. Samples needing more than 22 cycles may not contain enough cDNA for accurate analysis and may be an indication of poor sample quality and in such cases samples were excluded from the samples analyzed. The quality and homogeneity of the cDNA used for quantitative PCR was established by amplification of 18S rRNA and the reaction products were evaluated by electrophoresis on agarose gel (1%)

6.4 Real-time quantitative PCR

In this thesis, Quantitative RT-PCR (qPCR) was used to compare the expression of a group of genes involved in the thyroid axis. Gene expression was compared between the different tissues analyzed, and between the experimental groups of fish treated with goitrogenic compounds and control fish.

6.4.1 Overview

Real time qPCR quantification is a method that has been introduced relatively recently and has found widespread use for applications such as allelic discrimination and expression profiling. A number of commercial companies have generated "ready to use reagents" although they tend to be very expensive which limits the application of this method. Moreover, most of the problems characteristic of the extreme sensitivity of RT-PCR get even more important in qPCR and caution must be exercised in order to avoid contamination.

Current detection methods are based on changes in fluorescence proportional to the increase in product, whether specific or none specific. Fluorescence is monitored during each PCR cycle to provide an amplification plot, allowing the user to follow the reaction,

and the threshold cycle (Ct, the cycle at which the fluorescence generated within a reaction crosses a threshold limit) is registered and compared for each sample or control reaction (fig. 8). The threshold is set in a level at which a statistically significant increase in fluorescence is first detected, when the amplification signal becomes higher than the fluorescence background, and associated with an exponential growth of PCR product in all samples.

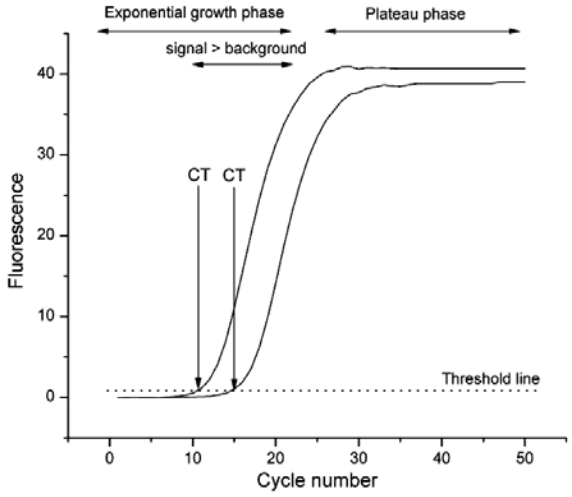


Figure 8 - Real-time qPCR response curves. Fluorescence is monitored during each PCR cycle to provide an amplification plot. The threshold cycle (Ct) corresponds to the cycle at which a statistically significant increase in fluorescence is first detected associated with an exponential growth of PCR product, and when the amplification signal becomes higher than the background of fluorescence. A threshold line should be set sufficiently above the background (from Kubista *et al*, 2006).

There are two types of chemical detection used in Real-Time qPCR: Non-specific detection, which monitors all double strand DNA produced during the reaction and specific detection, in which probes are used, which may be labeled with different dyes and due to their different excitation and emission spectra allow the dyes to be distinguished from one another. To the former group belong the TaqMan® probes, which were used to develop the assays established in the present thesis to quantify the expression of target genes.

The TaqMan® Probes, also called double-dye oligonucleotide or dual labeled probes, contain a reporter fluorophore attached to one end of the probe and a quencher fluorophore at the other end, which reduced the fluorescence of the reporter. The molecular events in a

Taqman real time qPCR (fig. 9) involve the following steps: 1) the probe binds to the amplicon during each annealing step of the PCR; 2) when the Taq polymerase extends from the primer bound to the amplicon it degraded the 5´end of the probe, which is then degraded by the 5´-3´exonuclease activity of Taq polymerase; 3) cleavage continues until the remaining probe melts off the amplicon. This process releases the reporter and quencher fluorophores into solution, spatially separating them compared to when they were held together by the probe. This leads to an irreversible increase in fluorescence from FAM and a decrease in the fluorescence of TAMRA.

Because the Taqman probes only hybridize with the specific target sequence in any given qPCR reaction and only when the probe is located in between the primers during the amplification, even if there is some specific secondary products being amplified they are unlikely to specifically bind the probe and therefore will not be detected, thus making this technique extremely specific and accurate.

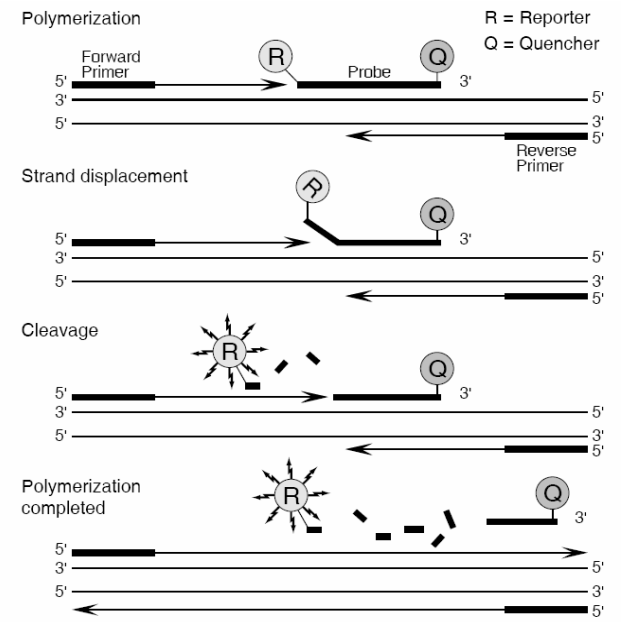


Figure 9 - Diagram depicting the molecular events in a TaqMan real-time qPCR in each amplification cycle. After the primers and TaqMan probe anneals with the target sequence, polymerisation begins. As a direct consequence of the 5' nuclease activity of the DNA polymerase used, and as polymerisation progresses, the TaqMan probe is first displaced and then broken-down liberating the reporter and quencher elements. As the reporter gets farther away from the quencher it starts to emit fluorescence which is then measured and used to calculate in real-time the quantity of PCR product accumulation. (from TaqMan Human endogenous control plate protocol - AppliedBiosystems)

Moreover, by using a standard curve composed of serial dilutions of the target sequence (from 1 fentomole to 1 nanomole) at the same time as samples are analysed an amplification curve can be constructed (fig. 10). This curve gives the amount of product amplified from a given concentration of starting material and using this curve the concentration of the target transcript in each sample can be quantified. Moreover, because of the quantitative nature of the method the expression of different genes can be directly compared. However, to permit inter tissue comparison the amplification curve obtained per target has to vary by less than 5%.

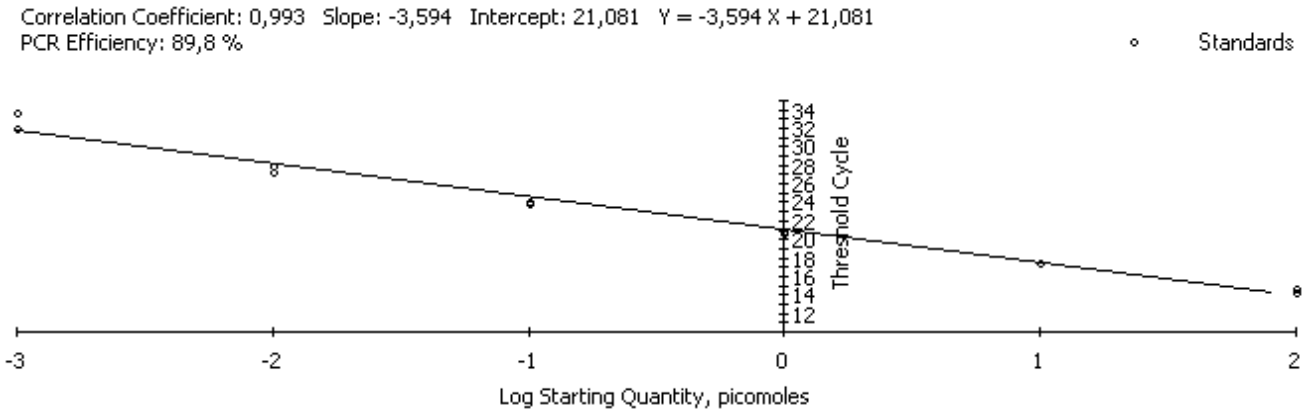


Figure 10 Standard Curve for the amplification of 18s gene detected using a FAM labeled probe. Blue points correspond to serial dilutions (10-fold dilutions from 1 ng/µl to 1 fg/µl) of the standards. Threshold cycle (Ct) corresponds to the cycle at which a statistically significant increase in fluorescence is first detected associated with an exponential growth of the PCR product.

Relative quantification, the most widely used technique, was used in the present study to measure gene expression and an endogenous control (18S ribosomal cDNA gene) was also amplified from the same cDNA as the gene of interest. All the qPCR reactions conducted in the present study were normalized with 18S ribosomal RNA to normalize for the amount of RNA used and for RT efficiency in the cDNA synthesis reaction of each sample (sample-to-sample variations). 18S was set as control, since theoretically it is a transcript with a fairly constant expression in different tissues.

6.4.2 RT-qPCR optimization

The specific Taqman probes and primers for each gene analyzed (sea bream TSHβ, Deiodinases I, II, and III, TTR, TRα and TR-β) were designed using the original sea bream sequences using Beacon Designer software (Premier Biosoft International, Palo Alto, CA, USA). Sequences of both, primers and probes from the genes expressed in higher quantities are represented in fig. 11. The probes were labelled at the 5'-end with FAM and at the 3'-end with TAMRA-6-FAM (Thermo Fisher Scientific,MA,USA). The concentration of probe and primers was optimized testing different concentrations (50, 100, 300 nM for the probe; and 50, 300, 500, 900 nM for the primers), using the below described reaction conditions and analyzed with Bio-Rad iCycler iQ qPCR thermocycler and software (Bio-Rad Laboratories). The optimal concentrations were established using the following criteria: highest fluorescence, best log fit and linear phase and lowest Ct value.

Gene	Kb	Primers and probe sequence
18s	134	316 TTT CGA TGG TAC TTT CTG TGC CTA CCA TGG TGA CCA CGG GTA ACG 361 GGG AAT CAG GGT TCG ATT CCG GAG AGG GAG CCT GAG AAA CGG CTA 406 CCA CAT CCA AGG AAG GCA GCA GGC GCG CAA ATT ACC CAC TCC CGA 451 CTC GGG GAG GTA GTG ACG AAA AAT AAC AAT ACA GGA CTC TTT CGA 496 GGC CCT GTA ATT GGA ATG AGT ACA CTT TAA ATC CTT TAA CGA GGA
DI	129	226 TTT GGC TCG AAA GAG TTC ATC AAA ACC GCC TCC CAT CAC TTG TGG 271 ATG TCT CTA GGA CAA GAG GCT TTT GTG GGA GGC AAA GCG CCA GAC 316 TCG CCT GTG GTC ACC ATG AAG GGG GAG ACA AAG AAC ATC TGC AAG 361 TAT TTA AAC GGC AAC AGG CCG CTG GTG CTG AGT TTT GGA AGT TGC
DII	101	1 TTC GGT TCG GCC ACC TGA CCC CCC TTC ATC AGC CAC CTG CCA GCT 46 TTC CGG CAG TTG GTT GAG GAC TTC AGT GAT GTC GCT GAT TTC CTG 91 TTA GTG TAC ATT GAT GAG GCT CAC CCA TCT GAT GGC TGG GTA GCC 136 CCT CCT ATG GGC TCT TGC TCT TTC AAT GTC CGG AAA CAT CAG AAC
TTR	78	316 AAA GCT TAC TGG ACG AAT CAG GGC AGC ACG CCG TTC CAT GAA GTA 361 GCT GAG GTG GTG TTT GAC GCC CAT CCT GAA GGC CAT CGT CAC TAC 406 ACC TTG GCT CTG CTG CTC AGT CCG TTC TCC TAC ACC ACC ACC GCC 451 GTG GTC TCC AGC GTA CAC GAG TGA CCC GCG TCA CAC TGA GAT CTT
TRβ	97	1261 TAC ATC AAT TAC CGC AAA CAC AAA GTG GCA CAT TTC TGG CCA AAG 1306 CTG CTA ATG AAG GTG ACG GAC CTG CGG ATG ATC GGT GCC TGC CAC 1351 GCA AGC CGA TTC CTC CAC ATG AAA GTT GAG TGT TCC ACC GAG TTA 1396 TTC CCT CCT CTC TTC CTA GAG GTC TTC GAG GAC TGA CCA AAC GGA

Figure 11. Correct probes (boxes) and primers (boxes with arrows) for each gene analyzed (sea bream 18S, Deiodinases I, II, TTR, TR-β) and amplicon length (Kb - base pairs) designed using Beacon Designer software (Premier Biosoft International). TRα, TSHβ and deiodinase III probes and primers not shown.

6.4.3 RT-qPCR conditions

Real time RT-PCR reactions were performed using Eppendorf qPCR mastermix 2,5X (eppendorf, UK) in a 25 µl reaction volume and 1 µl of sample cDNA. The thermocycle utilized was 1.5 min at 95 ºC followed by 45 cycles of 15 seconds at 95 ºC (denaturing) and 1 minute at 60 ºC (combined annealing and extension). Quantification was carried out using the standard curve method with serial dilutions (10-fold dilutions from 1 ng/µl to 1 fg/µl) of plasmid DNA containing the cloned target gene in each experiment, previously quantified using a commercial kit (Quant-iT DNA Assay Kit and Qubit fluorometer, (Invitrogen, Carlsbad, CA. USA). Since a requisite for quantification of unknown samples by the standard curve method is that the number of transcripts of each gene should fall on the linear part of the standard curve (between the lower and higher values), cDNA sample dilutions were also tested. Results were exported to an Excel spreadsheet containing columns with the sample well number, Ct value and starting quantity logarithm and an XY (scatter) plot was drawn with the log product concentration per cycle against Ct (similar to that shown in Figure 9). The template starting concentration was calculated from the Ct values of each sample utilizing the standard curve parameters (correlation coefficient, slope, and intercept with Y axis). To normalize the quantity of each amplified target transcript, the concentration of target amplicons obtained per reaction were divided by the values obtained of the 18S gene.

7. Statistical analysis

Statistical analyses were performed using the statistical software package Sigma Stat version 3 (SPSS, Chicago, USA). The results of gene expression from the four experimental groups were tested using one-way analysis of variance (ANOVA) and Tukey multiple comparison test, to test if significant differences were found between treatments. Differences were considered significant at $p< 0.05$. The results of T3 and T4 levels in plasma of all groups were tested using T-test and T3 and T4 levels from each fish were compared.

III. RESULTS

I thank Engª Nádia Silva for the histological analysis and Dr Marco A. Campinho for his advice and help in the Lowry assay, radioimmunoassay and Real-time qPCR.

1. Biochemical results

TTR and total serum proteins

TTR and total serum protein concentrations for each of the experimental groups are presented in fig. 12 and table 1. The concentrations of total protein measured for the control group was similar to concentrations previously determined for sea bream (Morgado et al., 2007b). No significant differences were found in the concentration of plasma protein in control versus chemically treated fish ($p > 0.05$). TTR concentration was measured by Western blot coupled to chemioluminiscence using 1 µl of serum from four fish of each group (fig. 13; table 1). One principal band of approximately 14 Kilo Daltons (KDa) predicted from the sequence and obtained in previous assays was detected by the anti-sbTTR sera. The quantification of the bands trough standard curve method show that concentration in the groups submitted to the goitrogenic compounds was not significantly different in comparison to the control group ($p > 0.05$) (fig. 11). The concentrations measured by this method in the present study were higher than values previously reported by Morgado (2007b) (3,8 ± 0,05 ng /µl), measured by ELISA.

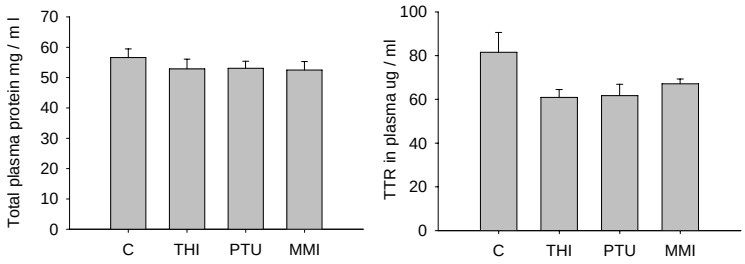


Figure 12 - Total plasma protein concentration (µg/µl) measured by Lowry assay in each group of fish (n = 7) and TTR plasma concentration (ng/µl) measured by western blot in each group of fish (n=4) at the end of the experiment. Data correspond to mean ± SE. No significant alteration was found in the concentration of plasma proteins or TTR in any of the chemically treated or control fish ($p > 0.05$). C – control; THI – thiourea; MMI – methimazole.

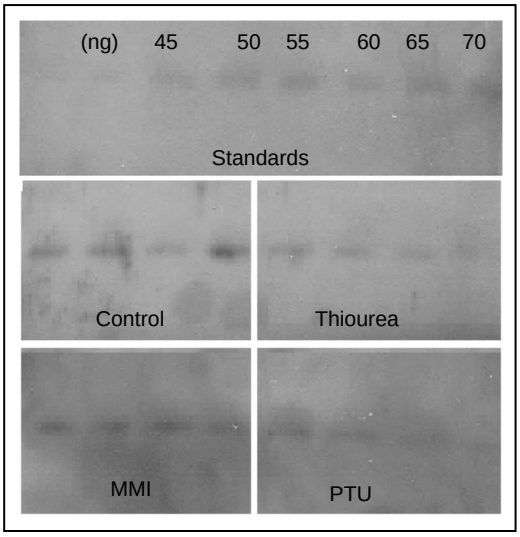


Figure 13 Western blot analysis with sbrTTR antisera (1:10000 dilution) of plasma samples from sea bream exposed to different goitrogenic compounds (B to E), and TTR standard curve from 45 to 70 ng (A). The blot was developed by chemioluminiscence and autoradiograph was exposed by 5 min. The same sample volume (1 µl) of plasma from each fish was loaded on the gel (n=4). -control group; thiourea; methimazole e PTU.

1.1 Thyroid hormones concentration

The concentration of T3 and T4 in fish plasma determined by RIA (fig. 14; table 1) revealed that after 21 days of exposure to MMI a significant decrease ($p < 0.05$) in T3 plasma concentration in relation to the control and the other groups, although no significant changes in plasma T3 concentrations were found in thiourea and PTU treated fish in relation to the control fish ($p > 0.05$). The concentration of plasma T4 showed a similar pattern to that observed for T3. Interestingly, T4 concentration in the MMI treated fish was to low and undetectable by RIA. The concentration of T4 in the thiourea and PTU treated fish was not significantly different to the control fish ($p > 0.05$). Comparison between T4 and T3 leves in each group, showed that in PTU and thiourea treated fish and also in the control group, T3 concentrations were not significantly different of T4 concentrations ($p > 0.05$) and therefore the plasma T3/T4 ratio, which is a sensitive index of change in thyroidal status was not altered.

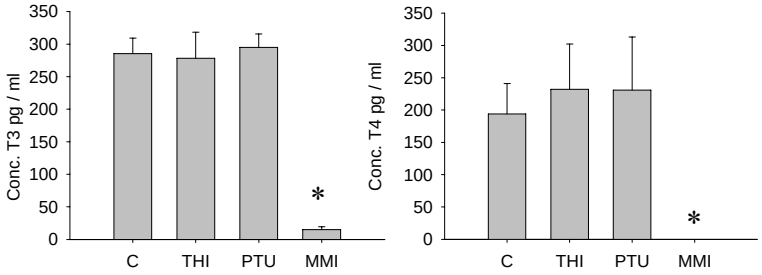


Figure 14 Concentration of plasma (pg/ml) of T3 and T4 levels, measured by RIA in each experimental group. C- control group; THI – thiouracil treatment (21 µg/mg fish); MMI – methimazole treatment (21 µg/mg fish); PTU- PTU treatment (21 µg/mg fish). Only MMI treatment caused a significant decrease in circulating T3 and T4 levels. Data correspond to mean ± SE (n=6). Differences from corresponding controls are marked with * ($p<0.05$).

Table 1. Concentration of total plasma protein, circulating THs and TTR in each of the experimental groups.

Treatment	T4 (pg ml -1)	T3 (pg ml -1)	Total protein (µg µl-1)	TTR (ng µl -1)
Control	194± 46	285,38 ± 23	56,6 ± 2,85	88,67 ± 12,27
Thiourea	232 ± 79	278,2 ± 40	52,83 ± 3,23	55,22 ± 6,27
Methimazole	-	15 ± 4	52,49 ± 2,78	87,86 ± 5,90
PTU	230,9 ± 82	294,9 ± 20	53,08 ± 2,33	78,09 ± 9,97

2. Histology of thyroid follicles

Examination of the thyroid tissue from the different groups of fish (Fig. 15) revealed that in control fish the follicles had a typical morphology, in which a cuboidal follicular epithelium (stained blue) surrounded an intensely staining acidophilic colloid (orange colour). The colloid in the lumen of some of the follicles contained occasional vacuoles characteristic of normal thyroid activity. The morphology of thyroid tissue in MMI treated fish was drastically different from the fish in the other treatment groups and the control. Most of the follicles are collapsed and no lumen is evident in a number of follicles. The morphology of the follicular epithelial cells (thyrocytes) is indicative of hypertrophy and hyperplasia as the cells are columnar and invaginations of the epithelium are evident (arrow). There is a striking change in the affinity of thyrocytes for the pigments used in histology and instead of staining dark blue the cytoplasm of these cells is stained red. The follicles that are not collapsed possess very little colloid which is full of vacuoles. Intracellular colloid droplets are present in thyrocytes of all the follicles and probably accounts for the altered staining

characteristics of these cells; this feature is not found in any of the other treatments. All of these characteristics are indicative of altered thyroid activity.

In PTU treated fish two classes of follicles are present; i) big follicles which have a cuboidal epithelium and are filled with colloid, similar to what is seen in control fish and indicating normal activity, and ii) hyperplastic follicles identified by the presence of invaginations in the follicular cell epithelium (arrow) and the almost complete absence of the follicle lumen. As is observed in the MMI treatment the hyperplastic epithelial cells are also hypertrophic as their epithelium is columnar and colloid is absent or has become basophilic and stain light blue (asterisk), denoting the absence of hormone. The thyroid tissue in thiourea treated fish has a similar morphology to the control fish and the main difference is the apparent increase in the number of follicles and also proliferation of interstitial tissue was also apparent.

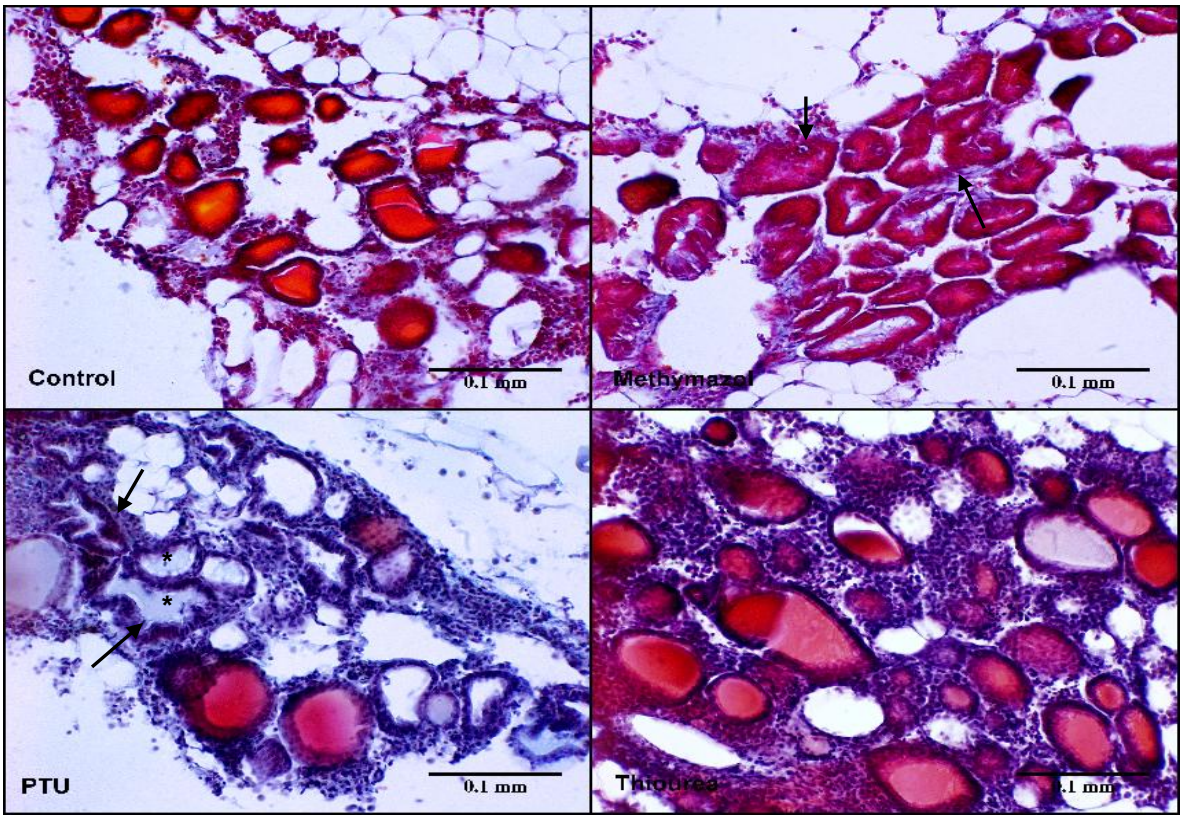


Figure 15 Cleveland-Wolfe staining of sections of sea bream thyroid tissue from control, MMI, PTU and thiourea treated fish. Note the intensely red staining colloid in the follicle lumen which with the exception of thyrocytes in the MMI treated fish is surrounded by a single layer of blue staining thyrocytes. * - basophilic colloid, Arrows - hyperplasic and hypertrophic follicles. Scale bar 0,1mm. Image provided by Eng. Nádia Silva.

3. Molecular results

3.1 Quality of mRNA and cDNA synthesis

The quality of the extracted RNA from liver and kidney samples used for RT-PCR was ensured by measuring its absorbance at 260 and 280 nm (1Abs260=40µg/ml of RNA; ratio Abs260/Abs280 estimates RNA purity) and analyzing 1 µl of RNA by agarose gel electrophoresis. In order to keep the necessary amount of RNA for the cDNA synthesis, this procedure was not developed for the extracted RNA from pituitaries. Low degradation and contamination of genomic DNA were observed for the RNA extracted from liver and kidney in the agarose gel (fig. 16).

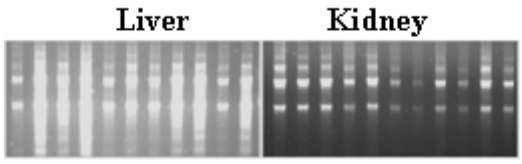


Figure 16 Ethidium bromide stained 1 % Agarose gel on which 1 µl of RNA extracted from liver (A) and kidney (B) was fractionated by electrophoresis. Note that two principal bands are visible which correspond to 28S and 18S rRNA. The absence of nucleic acids on the lower part of the gel is indicative of the absence of sample degradation and resulting production of low molecular weight products. In photograph (A) RNA loading is much higher and gives a more diffuse pattern than in B.

All samples from pituitary, liver and kidney were quantified with a commercial kit by fluorimetry using 1 µl of RNA, and 500 ng were treated with DNase and used for cDNA synthesis. The quality of cDNAs was assessed by amplification of 18S rRNA by semi-quantative RT-PCR (fig. 17) and also by RT-qPCR. Typically, the 18S rRNA qPCR assay yields Ct values $\leq$ 22. Samples producing Ct values far above 22 may not contain enough cDNA for accurate analysis and in this case such samples were excluded from the analysis.

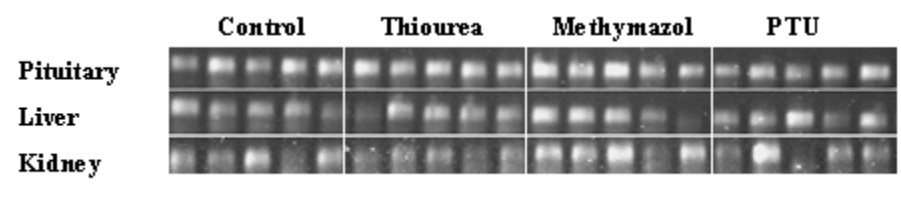


Figure 17 Ethidium bromide stained agarose gel (1%) on which the reaction products of 18S rRNA were fractionated by electrophoresis. The reaction products of 18s rRNA PCR reactions from 5 individual samples per treatment group is shown for pituitary, liver and kidney samples.

3.2 Real-time qPCR optimization

Primer optimization for each gene was carried out utilizing 900 nM, 500 nM, 300 nM and 50 nM dilutions (concentration in the PCR reaction) of forward and reverse primers, 100 nM dilution of the probe and minipreps with each clones genes which had been previously quantified. All the possible combinations between primers (concentrations) were tested and according to the Real-time amplification curves (see Fig. 9 in the 6.4 section of the methods) the primer pairs with the best log fit, fluorescence and lowest threshold cycle (Ct) were selected. After primer optimization, probe optimization was carried out using a range of different probe concentrations, 300 nM, 100 nM and 50 nM. Optimal concentrations selected for both primers (forward - reverse) and probes are presented in table 2, together with the sequence of all primers and probes.

Table 2. Concentration and sequence of primers and probes used to quantify the gene expression by Real-time qPCR

Gene	Sequence forward primer qPCR concentration (nM)	Sequence TaqMan probe qPCR concentration (nM)	Sequence Reverse primer qPCR concentration (nM)
DI	5'- TCG ATG GTA CTT TCT GTG CCT AC-3' 50	5´- CGC CAG ACT CGC CTG TGG TCA CC-3´ 50	5´- CAA CTT CCA AAA CTC AGC ACC AG-3´ 300
DII	5'-GGT TGA GGA CTT CAG TGA TG -3' 300	5'-TTA GTG TAC ATT GAT GAG GCT CAC CCA-3 100	5'-GAA AGA GCA AGA GCC CAT AG-3' 900
DIII	5'-CTA TCG GCA GGG GAA GAG ATG-3' 50	5'-CGG CGG TGT CCA AAT GGC GAG GG-3' 100	5'-CAC AGG GCT GCA TT TTC AG-3' 900
TSHβ	5'-TCG GTG TAC CAA ACC AGT CAG-3' 900	5'-ACC CGT ATC CCG ACC AGA GCA ACT-3' 100	5'-GCG CTA ACA ACAGGA AGA TCA G-3' 900
TTR	5'-GTC ACT ACA CCT TGG CTC TGC-3' 900	5'-CGT TCT CCT ACA CCA CCA CCG CCG-3' 100	5'-GTC ACT CGT GTA CGC TGG AG-3' 900
TRβ	5'-CAA AGC TGC TAA TGA AGG TGA CG -3 300	5'-CCT GCG GAT CGG TGC CTG CC-3' 100	5'-GAA TAA CTC GGT GGA ACA CTC AAC -3' 900
18s	5'-TCG ATG GTA CTT TCT CTG CCT AC-3' 300	5'-CAC ATC CAA GGA AGG CAG CAG GCG-3' 100	5'-AGT CGG GAG TGG GTA ATT TGC-3' 900

Quantification was carried out using the standard curve method with serial dilutions (10-fold dilutions from 1 ng/µl to 1 fg/µl) of plasmid DNA containing the cloned cDNA for each of the target genes, included in all plates analysed. The quantification of unknown samples was done using sample dilutions (1:2 for all the target genes and 1:10000 dilution of 18s rRNA) in order to obtain transcript levels on the linear part of the standard curve.

3.3 Gene expression

The effect of the goitrogenic compounds used in the study on the Sea bream thyroid axis was assessed at various levels (TH biosynthesis, transport, and metabolism) by analyzing expression of target genes associated with specific functions in the thyroid axis in real-time qRT-PCR experiments. Transcript expression corresponds to relative units and normalization was ensured by dividing by absolute values of 18s rRNA expression.

3.3.1 Quantification of target transcripts in liver, pituitary and kidney of control fish

The standard curve is extremely informative about the assay as it can give indication of PCR efficiency and sensitivity and also errors arising from factors such as pippeting. The slope of the standard curve was used to determine the exponential amplification and efficiency of the PCR reaction (100 % efficiency corresponds to a slop of -3.32). Before analyzing the effect in thyroid gene expression of each goitrogenic compound, the relative expression of target transcripts in liver, pituitary and kidney was analyzed in control fish (table 3).

In the pituitary of control fish higher expression of TSH β-subunit (3,4E-02 ± 6,1E-03) was observed in comparison to relative expression of TRβ (1,9E-0,6 ± 6,4E-07). TTR was the most expressed gene in liver of control fish (5,7E-0,3 ± 1,1 E-03) followed by deiodinases expression (3,3E-04 ± 9,6E-0,5 DII; 1,2E-07 ± 5,5E-0,8 DI). In addition, very low levels of DII expression in pituitary and kidney, DI in pituitary and DIII in liver were observed but outside of the qPCR standard curve.

Table 3.Relative expression of target genes in control fish, PCR efficiency and correlation coefficient of standard curve. 18s values are represented in pg and values corresponding to transcript number of thyroid genes were normalizing by dividing by absolute values of 18s rRNA expression and are represented in relative units.

Tisssue	Gene	Relative expression In control fish	PCR efficiency (%)	Correlation coefficient
Pituitary	18s	7,65E+02 ± 101,9	87.5	0.982
	TSHβ	3,4E-02 ± 6,1E-03	94	0.996
	TRβ	1,9E-0,6 ± 6,4E-07	105.2	0.999
	DII	(-)	90	0.996
Liver	18s	1,35E+03 ± 85,1	104.5	0.998
	TTR	5,7E-0,3 ± 1,1 E-03	96	0.994
	DI	1,2E-07 ± 5,5E-0,8	117.6	0.999
	DII	3,3E-04 ± 9,6E-0,5	100.4	0.998
	DIII	(-)	63.4	0,981
Kidney	18s	2,97E+03 ± 193,9	89.2	0.999
	DI	7,4e-8 ± 3,5e-8	111.2	0.999
	DII	(-)	92,9	0.993

- (-) indicates genes that were tested but expression was low and not quantified.

3.3.2 Quantification of target transcripts in liver, pituitary and kidney of the experimental and control fish

The analysis of transcript expression of TTR produced in liver (fig. 18) revealed that at the end of the experiment no significant alterations occurred between control fish and the fish treated with goitrogenic compounds ($p > 0.05$). In the liver, an organ which participates in the peripheral control of THs, the analysis of expression of deiodinase enzymes which are responsible for activation and inactivation of THs (fig. 17) revealed that DI and DII were strongly up-regulated by MMI treatment ($p < 0.05$) but not by any of the other goitrogenic compounds. DIII had an extremely low expression in the liver and it was not possible to quantify it or detect any changes due to the late cycle threshold of this assay. Analysis of pituitary expression of TSHβ, a hormone that stimulates the thyroid follicles to produce TH revealed that there was no significant difference ($p<0,05$) between the expression in control and groups treated with goitrogenic compounds (fig.18). Thyroid hormone receptors (TRs) function as transcription factors and directly regulate target gene expression through DNA response elements. Expression of TRβ in the pituitary was significantly decreased ($p < 0.05$)

in the fish treated with MMI (fig. 18). Expression of TRβ in the pituitary of fish treated with thiourea and PTU did not differ significantly from control fish. It was not possible to measure TRα as RT-qPCR development was not possible in the time available, it will clearly be of interest to measure it in the future to compare the relative importance of TRα and β in TH signaling at the level of the pituitary gland.

DI and DII expression was also assessed in the Kidney (fig. 18), but the levels of DII expression were so low it was not possible to reliably measure transcript expression in either control of treated fish. The expression of DI in the kidney was also low but was measurable. No significant differences were observed in DI expression between the kidney from control fish and from the fish which had received the goitrogenic compounds (fig. 19). However, with the exception of MMI the expression of DI was extremely variable, suggesting a differential response to the treatment by the fish.

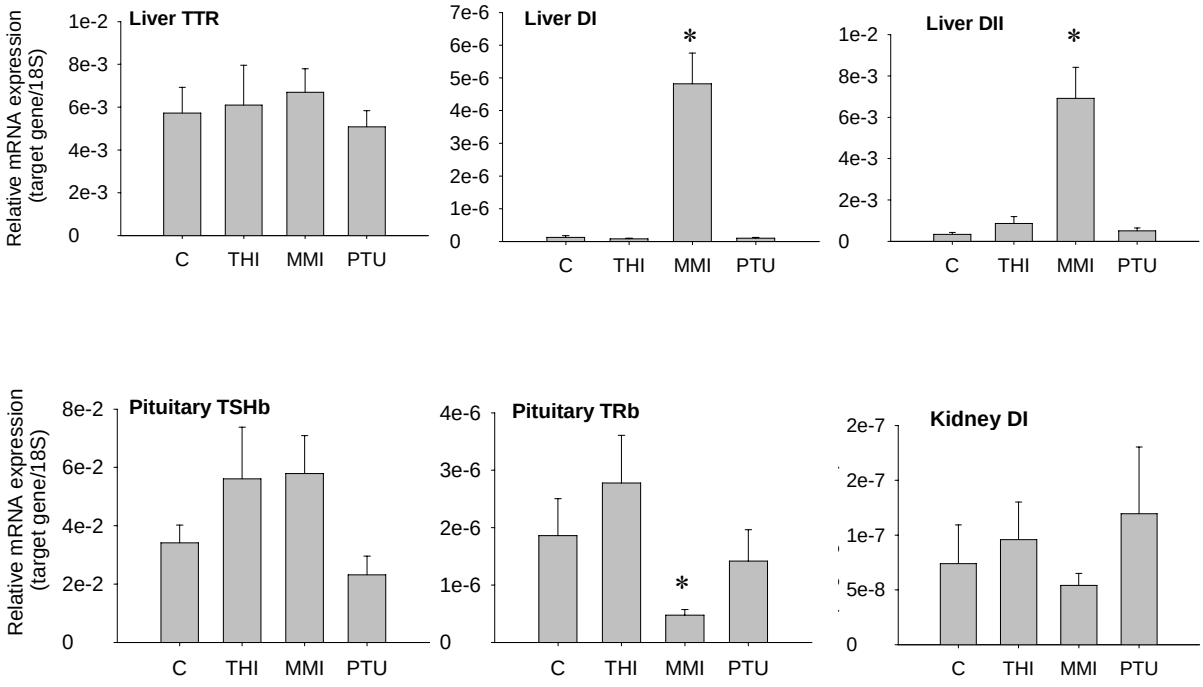


Figure 18 Quantitative real-time RT-PCR analysis of TTR, DI and DII gene expression in liver; TSHβ and TRβ gene expression in the pituitary and DI gene expression in kidney. C- control group; THI – thiouracil treatment (21 µg/mg fish); MMI – methimazole treatment (21 µg/mg fish); PTU- PTU treatment (21 µg/mg fish). Values corresponding to transcript number in the different samples were normalized by dividing by absolute values of 18s rRNA expression values and are represented in relative units. Data are presented as mean ± SE (n=6 or 5). Significant differences from corresponding controls are marked with * ($p<0.05$).

IV. DISCUSSION

The present study aimed to clarify whether well known human goitrogens can depress serum TH concentrations in fish and using histological techniques and gene expression studies better characterise the thyroid axis and its response to goitrogens in sea bream. The mode of action of the goitrogenic compounds used in the present study is already well characterised in mammals (http://www.thyroidmanager.org/, ; Gerber et al., 1981; Shadlinskii, 1999) and they have been widely employed in fish experiments over the past four decades. A number of groups have studied the effect of goitrogens on fish physiology, principally during metamorphosis and in larvae stages (Manzon et al., 2001; Matta et al., 2002; van der Ven et al., 2006) but the results have been variable.

T4 and T3 hormone levels in plasma combined specifically with histopathology analysis of thyroid follicles should be indicative of thyroid axis disruption in sea bream. The integration of such analyses should provide a specific pattern for antithyroid effects in this species and should permit correlation with changes in expression levels of genes involved in the thyroid axis.

In the present study T4 and T3 concentrations were not significantly different (T3= 285±23; T4= 194 ± 46 pg/ml) in plasma samples of control fish which is in contrast to the situation in mammals in which plasma T4 generally exceeds levels of T3 (Leatherland, 1994). The concentration of THs detected in sea bream is typical of a number of different species and the relative concentration of T4 to T3 may be related to deiodinases activity (Power et al., 2001). Moreover, plasma T3:T4 ratios can vary depending on the physiological status of the animal which may be associated with the time of day, ambient salinity, water temperature, pH levels and reproductive condition (Eales and Brown, 1993; Klaren et al., 2005).

Plasma T4 and T3 concentrations were only altered in sea bream treated with MMI and in these fish circulating levels of T3 decreased significantly and T4 was not detected by the RIA. Similar effects have been reported in tilapia in which 0,2 % MMI was administered in the food (Van der Geyten et al., 2001), although frequently administration of goitrogenic compound is not accompanied by a decrease in serum T4 levels or T3 levels (Van der Geyten et al., 2001; Elsalini and Rohr, 2003).

PTU and thiourea have been reported to reduce plasma TH levels in many teleosts (Brown, 1997; Swapna et al., 2006; van der Ven et al., 2006). Surprisingly, in present study PTU

and thiourea treatment did not cause a significant alteration in plasma TH levels, which were similar to those in control fish. This euthyroid state is consistent with the normal or marginally altered histological appearance of the thyroid tissue in the thiourea and PTU groups. The fact that these goitrogens failed to induce hypothyroidism is difficult to explain. However, a similar lack of effect has been recorded in a number of other studies with other species of fish. In fact, the results from studies investigating the effects of goitrogens on serum TH concentrations in fish are difficult to compare as a consequence of significant differences in the experimental protocols, route of administration, dose and time of exposure, and different state of development which tend to make the interpretation and comparison of the results difficult. The lack of effect found with PTU and thiourea treatment may be a consequence of several factors. For example, in the present study THs were analysed at a single time point and it is possible that initially the goitrogens may have disturbed thyroid axis homeostasis but that by the end of the experiment it may have been re-established. Alternatively, it has been shown that goitrogens act in a dose-related manner in mammals and fish [24, 46] as observed in rats, where plasma PTU levels in excess of 0.18 microgram/ml completely inhibited thyroid hormone synthesis, levels between 0.14 and 0.09 microgram/ml had a partial effect and levels less than 0.09 microgram/ml had no effect on thyroid hormone synthesis (Francis and Rennert, 1980). Moreover, in accordance to the initial doses used to control hyperthyroidism in humans lower doses of MMI (15-60 mg/day) compared to PTU (150-300 mg/ day) are used, and therefore it is possible that the dose administered in the present study (1 mg/kg day) may have been insufficient to cause hypothyroidism and in future it will be important to test if higher doses of PTU and thiourea have a goitrogenic effect.

It is clear that regulation of THs levels in plasma is the primary function of the brain - pituitary-thyroid axis in teleost fish, and other vertebrates, and the production of THs is controlled by feedback mechanism (Eales and Brown, 1993). TSH excretion is increased when low levels of TH are presents in the blood and the latter has been demonstrated to increase plasma TSH and pituitary TSH mRNA in hypothyroid fish (Larsen et al., 1997). However, in the present study sea bream TSH mRNA expression did not appear to be altered despite the pronounced hypothyroid status induced by MMI. Moreover, despite the apparent absence of change in pituitary TSH mRNA expression, the histological

appearance of the thyroid tissue was indicative of altered follicle activity normally associated with high levels of plasma TSH. This observation appears to suggest that there may be a mismatch between gene expression and TSH protein production and it will be important to establish if in the sea bream treated with MMI there is elevated plasma TSH. The mismatch between TSH plasma levels and gene expression may be indicative of the existence of a regulatory mechanism to reestablish normal TSH circulatory levels. Nevertheless such responses are difficult to evaluate and could be related to different times of response in thyroid axis endpoints.

The preliminary step in thyroid hormone formation is the attachment of iodine to tyrosyl residues in TG to produce MIT and DIT (see TH synthesis in topic 4 of the general introduction). Stored TG molecules undergo iodination and reactions driving hormone formation occur at the apical plasma membrane/lumen boundary where the peroxidase and H_2O_2 generating system resides. It is clear that iodide is oxidized by H_2O_2 and TPO, and transferred to the tyrosyl groups of TG and it is this step that the three major goitrogenic drugs (PTU, MMI and thiourea) affect in mammals. When reaching the thyroid tissue goitrogens appear to act by competing with tyrosyl residues of TG for oxidized iodine and this way block the production of THs (http://www.thyroidmanager.org/).

In general the turnover of intrafollicular material (colloid) varies greatly with gland activity and in the present study only MMI and PTU appeared to alter the thyroid follicles. TSH hyperstimulation causes several characteristic modifications in thyroid tissue: a) macropinocytosis is triggered and this leads to increased TG internalization by thyrocytes and a reduction in follicular TG content; b) there is an increase in thyroid tissue mitotic activity and a subsequent increase of cell number (Leatherland, 1994). An additional change in thyroid tissue appearance is associated with increased macropinicytosis, in which pseudopods developed from the apical plasma membrane of the thyrocyte project into the follicle lumen and pinch-off TG forming vacuoles known as colloid droplet. The delivery of the vacuole contents to lysosomes leads to an increase in the relative amount of endoplasmic reticulum and an associated increase in the size of the follicle cell (http://www.thyroidmanager.org/).

MMI appeared to have the same effect in sea bream as has been described in mammals. Stimulation under the presence of MMI and provably PTU leads to altered thyrocites

morphology as an attempt to compensate for reduced circulating hormone levels, where pharyngeal thyroid follicles exhibited signs of goitre such as hyperplasia and hypertrophy in the follicular epithelium, as has also been observed in rats (Gerber et al., 1981). The histological appearance of thyrocites in the follicles and their colloid droplet content suggests internalization of TG in the thyrocytes. This TG internalization together with the higher number of cells resulted in a reduction in follicle colloid content. It is surprising that MMI treated sea bream which had significantly increased thyroid tissue activity were unable to maintain plasma TH levels and in mammals this failure has been associated with the blockage of TPO's ability to activate iodine and transfer it to TG in MMI treated animals.

Although the morphology of the thyroid tissue was only drastically different for MMI treated fish, PTU also has a partial effect. In PTU treated fish normal follicles (similar to control group) and colloid-depleted follicles were observed, in addition to hyperplasia and hypertrophy of the thyrocites. However this effect was not strong enough to impair T4 production, and the depletion and accumulation of colloid in different cells suggest a reduction in iodine activation despite to the dose of PTU used in the present study. PTU is also known to increase the blood flow and produce hyperaemia in the thyroid area, in rats and fish (van der Ven et al., 2006), however in sea bream this phenomena was not analysed, and will be interesting to observe if PTU leads to increase in capillaries. A further factor may be the time-lag which can occur before the development of goiter, because of the reserves of hormones accrued during periods of iodine availability (Eales and Brown, 1993).

In the present study MMI and PTU were administrated in the same doses. That MMI had a stronger effect than PTU when administrated in the same concentration can be explained by the direct interaction of MMI with TPO's and the resulting irreversible inhibition which does not occur with PTU (Roy and Mugesh, 2005). Goitrogenic thiourea derivatives have been demonstrated to interfere in various ways with thyroid peroxidases (Freyberger and Ahr, 2006). In the present experiment no significant change in follicle morphology was observed although an increase in the number of thyrocytes occurred compared to control fish. The thyroidal impact of thiourea has been correlated in mammals with the time of exposure, dose and excretion rates (Hirate et al., 1982).

At the level of gene expression MMI treated fish was the only group that showed different levels of expression of genes involved in the thyroid axis compared to the control fish. For these reason the correlation between thyroid disruption and expression levels of genes involved in the thyroid axis will only be discussed for this group.

In order to evaluate the effect of goitrogens in different thyroid system endpoints, in addition to TSH expression the expression of other important genes was also measured. Relatively few studies have mapped the tissue distribution of deiodinases in fish and initially their distribution in key metabolic organs such as, the liver, kidney and pituitary of control fish was assayed, in addition to TTR in liver (the organ that produce TTR) and TRβ expression in the pituitary. Relative expression in control fish showed that in kidney as in liver DI was expressed at similar levels, but DII was mainly expressed in liver, being more expressed than DI. However extremely low DII expression levels were also observed in kidney and pituitary, but too low to be quantified by the qPCR. This result indicates the liver as a principal organ involved in peripheral deiodination in sea bream, being in accordance with published results for other teleosts (Mol et al., 1999).

In common with what occurs in mammals, fish liver deiodinases adjust to changes in TH plasma levels (Eales, 1990; Mol et al., 1999). However, in mammals in addition to their effect on the hypothalamic-pituitary-thyroid axis, PTU and MMI also inhibit DI expression. In the present study only MMI affected the deiodinases expression in sea bream. Van der Geyten, *et al.* 2001 observed in hypothyroid tilapias (also fed with MMI) a strong up-regulation of DI, which is barely expressed in euthyroid tilapia and also DII in the liver. The same effect is evident in the present study as the expression of DI and DII was up-regulated in the liver of fish with MMI induced hypothyroidism. Interestingly, in fish DI expression is not inhibited as occurs in mammals. Both DI and DII in fish transform T4 to T3, the biologically active form of THs (Eales and Brown, 1993), which suggests that an increase in these enzymes should help maintain normal T3 levels and therefore the processes regulated by these hormones. However, in situations of reduced T4 synthesis, production of T3 can eventually fail due to lack of substrate. Interestingly, although MMI caused an up regulation of DI and DII in the sea bream liver it had no effect in the kidney

and a similar observation has been made in the tilapia (Mol et al., 1999; Van der Geyten et al., 2001), suggesting that the regulation of these genes differs in the liver and kidney.

After peripheral regulation of THs by deiodinases enzymes, the cellular function of THs is ensured by TRs (Lazar, 1993). The THs have a small hydrophobic thyronine nucleus that mediates their action by binding to specific nuclear receptors, which act directly on target genes bringing about a cellular response (Yen and Chin, 1994). In the present study TRβ expression was downregulated in the pituitary of sea bream submitted to MMI treatment, suggesting that a decrease in circulating T3 levels changes TR expression and accordingly to the observed for a number of receptors, the ligand (T3) can directly regulate receptor expression, since the affinity to nuclear receptors is higher for T3, while T4 binds with low affinity and has few direct actions (Hadley, 1996; Darras et al., 1998). Therefore, the occupancy of nuclear receptors has been proposed to be controlled on the intracellular concentration of T3, which is influenced by a combination of the circulating T3 concentration and deiodinases activity. In addition, T3-TR binding can activate or repress TR-mediated gene transcription. Negative regulation of transcription by the TR is poorly understood. However, the promoters of several negatively regulated genes have been shown to be sufficient to confer T3-dependent repression. Several of the best studied examples involve genes in he hypothalamic-pituitary-thyroid axis that are subject to feedback inhibition by T3 (TRH, TSHa and TSHb) by interfering with assembly of the basal transcription complex (Lazar, 1993; Tagami et al., 1997). In this way negatively regulated genes are stimulated by unliganded TR and repressed upon the addition of T3. Until now, does not exist any report regarding TSH negative regulation by TR in fish. If this phenomenum was also observed in fish, TSH down regulation in sea bream could not be established by the TR and THs depletion**.** However the low T3 levels and low expression levels of TR observed for de MMI treated group did not altered TSH expression at the pituitary. Interestingly, targeted deletions in both alpha and beta receptor genes in mice, do not produce a syndrome analogous to the lack of thyroid hormones. The most likely explanation for the relative mild effects of receptor deficiency is that responsive genes are left in a "neutral" state, rather than being chronically suppressed as happens with hormone deficiency (Zhang and Lazar, 2000). However the function of TR varies between receptors

and their respective issoforms and in order to better understand the effect of MMI in TR levels will be important to analyzing also TRα expression.

In concerns to total protein and the THBP, no significant alterations were found in the concentration of total proteins and TTR in plasma in any of the goitrogen treated fish compared to control fish. The unchanged TTR concentration in plasma measured by Western was reflected by the same TTR transcript expression in liver in all the groups. Before the present study only one study has previously reported TTR concentrations in juvenile sea breams using ELISA (Morgado et al., 2007c), although the levels were approximately 20 times lower than those measure in control fish of the present study (control fish 88,6 ± 12 ng/µl – 0,16 % of total protein). The difference in the concentration measured may be related to differences in the method of analysis (ELISA versus Western blot), possibly differences in the standards utilised and also the physiological status of the fish. In the same study treatment of sea bream with MMI caused a significant increase in circulating TTR levels, although T4 and T3 plasma levels were not changed (Morgado et al., 2007c), although it is difficult to make direct comparison as the dose of MMI was significantly different as was the age of the fish utilised. It will be important to repeat analysis of the samples in the present study with ELISA which is a much more sensitive and accurate method of analysis.

V. Conclusions

- In conclusion, in sea bream MMI appears to operate in a similar manner to that in mammals, affecting the hypothalamus-pituitary-thyroid axis blocking the THs production probably by inhibiting TPO´s ability to activate iodine and transfer it to TG.
- The histological data of thyroid follicles in the present study and changes in plasma TH levels are in full agreement with the notion that MMI induced hypothyroidism in the sea bream by a similar mechanism to that observed in mammals.
- MMI could be a good experimental inducer of hypothyroidism *in vivo* in fish.
- PTU and thiourea fail to induce hypothyroidism, however their poor effect may reflect an inadequate dose rather than a fundamental difference in their activity in fish compared to mammals.
- The failure of this compound as antithyroid agent in related experiments could be related to levels of exposition and dose of administration, since until now no standardized method had been validated to assess thyroid disruption in fish.
- The alterations in deiodinases expression in response to hypothyroidism fits well the general notion that in the sea bream in common with other teleosts, peripheral deiodination is the primary control mechanism for regulation of T4/T3 levels in plasma, independent of the hypothalamus-pituitary-thyroid axis (Eales and Brown, 1993).
- In hypothyroid sea bream it was observed that a) liver DII exhibits reactions to hypothyroidism similar to those observed for most mammals c) liver DI in hypothyroidism reacts in an opposite manner that in mammals d) kidney DI is unresponsive to thyroid status. The results suggests that during hypothyroidism DII and DI in the liver increased in order to maintain plasma T3 levels.

V1. Future perspectives

The attribution of goitrogens effects to the thyroid function in fish is complex and for its study it is needed to consider numerous variables, some variables related to indirect and some to direct actions on the thyroid cascade. Therefore many factors should be considered when evaluating thyroid function impairment utilizing these compounds, specially the

doses of administration, the time of exposure and the route of administration. Additional research would be required before these compounds could efficiently be employed in thyroid screening. It will be important to improve further investigation in the thyroid axis of this species and clarify the effect of goitrogens in sea bream, since this teleost could be a model candidate for laboratory testing due to its easy availability and the large number of resources available (genomic libraries, cDNA libraries, microarrays etc.). In this context, it is necessary to investigate the effect of higher doses of PTU and thiourea and also evaluate different times of exposure in order to know the time of first appearance of the goiter and THs impairment.

As in sea bream thyroid axis MMI seems to operate in a similar manner compared to mammals, in future research it would be important to measure the TG content of thyroid follicles and TPO's activity *in vitro*. However, in the present study MMI appears to have little effect on TSH expression and will be important to observe the histological changes in the pituitary thyrotropes by himunnohistochemistry, in order to investigate possible alterations in protein TSH production and possible regulatory mechanisms to reestablish normal TSH circulatory levels.

MMI appears to have little effect on TTR production however the possibility that it could bind TTR and in this way disrupt the thyroid axis and affect TSH production should not be discarded. In fact previous studies have shown that TTR can bind many compounds that like MMI structurally resemble THs (Brown et al., 2004; Morgado et al., 2007a). In this context it would be important to repeat plasma TTR measurements by ELISA and analyse ligand binding between TTR and MMI.

Fish deiodinases exhibit discrete features that seem to be distinctive for piscine species. Indeed, DI responses to thyroidal status in teleosts differ from that exhibited by mammals. Nevertheless, current experimental evidence suggests that deiodinases may coordinate in a tissue-specific fashion the action of iodothyronines and in this context; future studies should compare tissue deiodinases expression and activity, especially between liver and kidney.

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VI. APPENDIX

Identity searches of putative estrogen-responsive genes, obtained in a previous study by suppression subtractive hybridization in the testis of sea bream (*Sparus auratus*)

Introduction

Estrogens are a group of steroid compounds enzimatically produced from androgens, and include the three major naturally occurring estrogens in women: 17β-estradiol (E_2), estriol and estrone. Estrogen's actions are mediated by ligation in the target tissues to specific nuclear receptors, the estrogen receptors (ER), or to plasma membrane localized receptors (Nilsson et al., 2001; Razandi et al., 2004). Binding to receptors, especially to the nuclear ERs, often results in the so-called "genomic actions", in which the transcription of estrogen-responsive genes (ERG's) is up or down-regulated, culminating in the estrogenic physiological effects; other effects may be mediated by rapid "non-genomic" actions, involving activation of cytoplasmatic signalling cascades and resulting in the activation of enzymes, ion channels and eventually also in the transcriptional activation of ERG's (Bjornstrom and Sjoberg, 2005; Levin, 2005).

Although estrogens are best known as key regulators of the reproductive system in female vertebrates, they also control a wide range of physiologic processes such as development, cell differentiation, bone metabolism, and the cardiovascular and central nervous systems (Barkhem et al., 2004). In fish, estradiol regulates female reproductive cycles, sex differentiation and the synthesis of egg yolk (specially vitellogenin) and egg-shell proteins in the liver, required for oocyte growth in the ovary (Flouriot et al., 1996; Celius et al., 2000; Lange et al., 2002).

However, there is now increasing evidence that low levels of estrogens are also important for normal male physiology (O'Donnell et al., 2001; Hess, 2003). In mammals, the disruption of ERα by gene knockout in mice or by treatment with antiestrogens resulted in decreased fertility, and there is also evidence for a role in the control of testis development and spermatogenesis in mammals (O'Donnell et al., 2001; Delbes et al., 2006), while in fish E_2 promoted stem cell renewal in huchen and in eel testis (Miura et al., 1999; Amer et al., 2001). Moreover, many disruptive effects on male fish physiology have been also attributed to estrogenic compounds found in the environment, acting as estrogen agonists or antagonists (Hutchinson et al., 2006).

In addition, testicular synthesis of E_2 has also been described in mammals, fish and amphibian (Varriale et al., 1986; Loomis and Thomas, 1999; Carreau et al., 2003), and the

expression of two nuclear ER subtypes in mammals and three in fish has also been detected in testis (including in sea bream, Pinto et al., 2006a).

In order to better understand the estrogen functions in male fish physiology, research concerning the identification of differentially expressed genes and the mechanisms is needed. However, in the last years an increasing number of ERG's have been accumulated in several species (e.g. O'Lone et al., 2004), although the induction mechanisms are in most cases unknown, and it is predicted that many remain to be identified.

Suppression subtractive cDNA hybridization (SSH) (Diatchenko et al., 1996) is a powerful tool for the identification and analysis of differentially expressed cDNAs. Recently, in our group (Molecular and Comparative Endocrinology), subtractive hybridization was used for the first time to isolate candidate ERGs in the testis of male sea bream treated with E_2, as a step to understand the mechanisms underlying normal or endocrine-disrupted testis physiology in fish (Pinto et al., 2006b). In this study, 183 clones obtained by subtractive library cloning were sequenced and are now available at NCBI with consecutive accession numbers from CX734847 to CX735033. Identity searches were carried out against non redundant public data-bases, and 129 of these sequences were identified as orthologous of known genes or ESTs, while 54 could not be identified (see below table 1). In addition, clones matching known genes were classified by biological process according to the Gene Ontology Consortium rules (see table 2-3). However, the proportion of clones matching known genes (29%) was relatively low and an update of this annotation could contribute to better understand the results of this experiment.

Recently, the Molecular and Comparative Endocrinology group added to the Marine Genomics European Network (MGE, http://www.marine-genomics-europe.org), and we were able to make use of high-throughput genomics data from several important research centers in Europe, including sea bream and sea bass ESTs (expressed sequence tags) libraries. The objective of the present work is to update the annotation of the sequenced sea bream E_2-treated testis subtractive library clones (Pinto et al., 2006b), by sequence comparison with the annotated EST libraries from sea bream and sea bass to which we have now access (MGE-EST Libraries) or by identity searches with updated public databases.

Methods

Identity searches of the 183 clones obtained by SSH in the previous study (Pinto et al., 2006b, accession numbers CX734847 to CX735033) were carried out using BlastX and BlastN (Altschul et al., 1997) against the public databases at http://www.ncbi.nlm.nih.gov/BLAST/. In addition, the 183 sequences were also compared by BlastX and BlastN to the sea bream and sea bass MGE-EST-libraries (at http://est.molgen.mpg.de/FishShellfish site blast MGE, restricted use to MGE members), and their identities identified by looking at the annotated identities of each MGE clones/contigs, previously obtained by identity searches with public available databases. Only matches with expected values (E)<10^{-5} (between SSH clones and public sequences/sea bream or sea bass ESTs or contigs) were considered significant. New genes identified were classified by biological process according to the Gene Ontology Consortium rules (Ashburner et al., 2000) through searches in the GeneCards webpage (http://bioinformatics.weizmann.ac.il/cards). Also, the identification of genes previously determined to be estrogen responsive in other tissues and/or organisms was carried out by searches in the ERG database (http://research.i2r.a-star.edu.sg/promoter/Ergdb-v11/index.htm).

Results

Identity searches resulted in the annotation of 23 additional clones clones (table 1), previously classified as unnamed or unidentified (Pinto et al., 2006b), including 15 clones which now matched known genes and 8 clones matching unnamed proteins. The identity of 7 clones was identified through blast against public data bases (NCBI) (6 matched known genes while one matched one unnamed protein) and 13 clones where identified through blast against sea bream MGE_EST libraries, corresponding to 7 known genes and 6 unnamed proteins. Identity searches through sea bass libraries also allowed the annotation of three clones, matching two known genes and one unnamed protein.

Table 1 - Summary of the results from Blast searches with the clones previously identified as unnamed proteins or unidentified, against public sequences (NCBI) or against sequences from Sea bream and Sea bass EST libraries available inside the Marine Genomics Network (MGE-EST Libraries).

Resource	Number of clones with significant match ($E<10^{-5}$)	Clones matching known genes	Clones matching unnamed proteins
NCBI	7	6	1
Sea bream library	13	7	6
Sea bass library	3	2	1
Total	23	15	8

The number of clones identified in 2006 and the number of clones identified in the present analyses are represented in table 2 and figure 1. 15 clones were annotated that matched known genes, whereas 8 unnamed proteins where classified as known genes. In addition, 8 clones previously unidentified were classified as unnamed proteins.

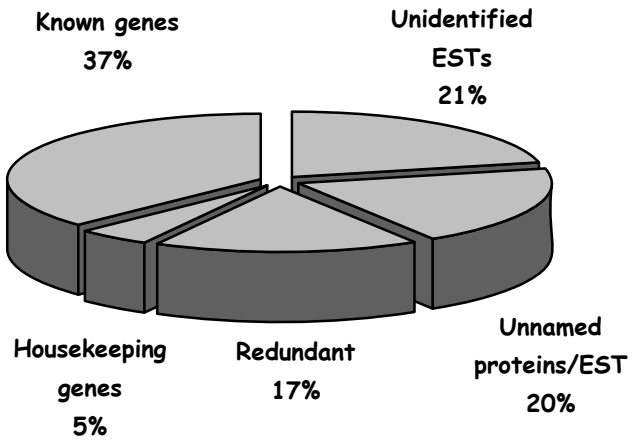

Figure 1 - Summary of the results from Blast searches with the 183 clones isolated from the E_2-treated testis subtractive library. Values are the percentage (%) of sequences.

Table 2 - Summary of the results from Blast searches with the 183 clones isolated from the E_2-treated testis subtractive library. Values are the number (N) of sequences within each group.

BLAST results	**N before**	N updated
Unidentified ESTs	**54**	39
Identified	**129**	144
Unnamed proteins/ESTs	36	**36**
Redundant	31	**31**
Housekeeping genes	9	**9**
Known genes	**53**	68
Total	183	183

Values are the number (N) of sequences within each group.

The 15 clones matching known genes were classified by biological process according to the Gene Ontology Consortium rules, increasing the number of candidate ERGs already classified in the previous analysis (table 3). Figure2 and Table 4 shows distribution of identified genes by biological process categories in the previous and in the present analysis, while it was observed in the previous analysis that most of the known genes were important in protein metabolism, sexual reproduction, cell proliferation, lipid metabolism, signal transduction and transport (Pinto et al., 2006b), this global pattern is still maintained after the new analysis, which increase mainly the number of genes involved in protein metabolism. A small increase was also verified for the categories: sexual reproduction, lipid metabolism, transport, energy pathways and nucleic acid metabolism. However 6 clones matched genes for which there is not yet information about the biological process in which they can involve. In addition, thee identification of the 15 new clones annotated as ERGs in this approach (see table 3) were not described to be estrogen responsive in other tissues and/or organisms through searches in the ERG database.

Table 3 - Classification of identified genes according to gene ontology terms in previous analyses through public data bases and in the present analysis, highlighted in bold; trough NCBI and sea bream and sea bass MGE-EST-libraries.

Accession Numbers	Identity[a]	Best match NCBI[b]	Best match MGs[c]	E[d]	F[e]	ERG[f]
1- Development- Sexual reproduction [GO:0019953]						
CX734849, CX734852, CX734878, CX734955, CX734956, CX734964, CX734975, CX735032	Vitellogenin II (VgII)	BAB79591		1E-56	8	Y
CX734983, CX734994	Vitellogenin I (VgI)	BAC20186		5E-45	2	Y
CX734862, CX734876, CX734911, CX734952, CX734987, CX734996, CX734999	Choriogenin L (ChgL)	CAA63709		1E-103	7	Y
CX734959, CX734963	Choriogenin H (ChgH)	BAA13994		5E-06	2	Y
CX734907, CX734970	Choriogenin Hminor (ChgHm)	CAA04220		2E-65	2	
CX734850	Egg envelope component ZPB (ZPB)	AAM91820		3E-24	1	
CX734851	Sperm associated antigen 1 (Spag1)/ infertility-related sperm protein	NP_003105		6E-33	1	
CX735020, CX735021	Egg envelope component ZPAX (ZPAX)	AAD38904		4E-97	1	
CX734947	Vitellogenin Receptor (VgR)	AAO92396		3E-06	1	
CX734982	**cd9 protein.**		**Seabream_2006-01-03-CL855Contig1**	**4,72E-145**		
2- Lipid metabolism [GO:0006629]						
CX734859, CX734867, CX734899, CX734914, CX734953,	Apolipoprotein A-I (ApoaI)	O42175		2E-59	13	Y

Accession Numbers	Identity[a]	Best match NCBI[b]	Best match MGs[c]	E[d]	F[e]	ERG[f]
CX734954, CX734957, CX734979, CX734997, CX735008, CX735017, CX735019, CX735026						
CX734856	Mitochondrial carnitine-acylcarnitine translocase (Slc25a20)	NP_065266		3E-53	1	
CX734897	Long-chain-fatty-acid-CoA ligase 1 (acsl1)	Q9JID6		9E-42	1	
CX734946	Long-chain-fatty-acid-CoA ligase 5 (acsl5)	Q8JZR0		2E-37	1	
CX734998	Long-chain L-2-hydroxy acid oxidase (Hao2)	NP_057611		1E-06	1	
CX734968	Oxysterol-binding protein-like protein 7 (Osbpl7)	AAL40659		1E-18	1	
CX735004	Delta-9-desaturase 2 (Ech1) *	CN981038		6E-10	1	
CX734967	**oxysterol binding protein-like 6 (osbpl6)**	**NM_001005927.**		**9E-34**		
3- Protein metabolism [GO:0019538]						
Protein biosynthesis [GO:0006412]						
CX734853	Ribosomal protein L13a (Rpl13a)	NP_997949		6E-12	1	
CX734960	Seryl-aminoacyl-tRNA synthetase 1 (Sars1)	AAH08612		3E-34	1	
CX734903	40S ribosomal protein S11 (Rps11)	AAG22825		2E-20	1	
CX734958	Ribosomal protein L17 (Rpl17)	AAF61071		2E-15	1	
CX735007	**eukaryotic translation initiation factor 4 gamma, 2 variant (IF4G2_HUMAN)**		**cDN11P0004F15.F.ab1**	**2,78E-74**		
	Protein catabolism [GO:0030163]					
CX734922	Trypsinogen-like serine protease *	AF134323		2E-36	1	
CX734909	Proteasome subunit beta type 8 (Psmb8) / Low molecular weight polypeptide 7	AAL37206		4E-24	1	Y
CX734937	Proteasome subunit beta type 6 (Psmb6) *	AY190669		1E-71	1	
CX734950	Cysteine proteinase (CysP)	AAB82743		4E-78	1	

Accession Numbers	Identity[a]	Best match NCBI[b]	Best match MGs[c]	E[d]	F[e]	ERG[f]
Protein modification [GO:0006464]						
CX734924	Protein arginine N-methyltransferase 3 (Hrmt1l3)	NP_005779		1E-30	1	
CX734991	Serine/threonine-protein kinase / Vaccinia-related kinase 1 (Vrk1)	NP_998550		8E-41	1	
CX734923	Cyclophilin A / peptidylprolyl isomerase A (Ppia)	AAR11779		6E-43	1	Y
CX735033	Dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1A (Dyrk1a) *	NM_001396		4E-24	1	
CX734980	**phosphoprotein phosphatase 2a-alpha catalytic chain (Ppp2ca)**		**cDN01P0006I08.F.ab1**	**1,10E-20**		
CX734977	**serum lectin isoform 2 (LMAN2)**		**Seabream_2006-01-03-CL2588Contig1**	**6,03E-47**		
CX734920	**Tubulin folding cofactor B (TBCB)**		**SeabassC-CL257Contig1**	**3,00E-60**		
4- Nucleic acid metabolism [GO:0006139] and mRNA processing (GO:0006397)						
CX734895	WD repeat protein Gemin5	AAL38980		6E-46	1	
CX734973	IMP cyclohydrolase (Atic)	NP_112276		7E-59	1	
CX734984	Nuclear cap binding protein subunit 2 (ncbp2)	NP_775356		4E-53	1	
CX734927	**stem-loop binding protein (slbp)**		**Seabream_2006-01-03-CL2821Contig1**	**1,89E-129**		
5- Transport [GO:0006810]						
CX734966	Translocating chain-associating membrane protein (Tram)	NP_705955		1E-66	1	
CX734945	Signal recognition particle 9kDa (Srp9)	NP_957390		1E-12	1	
CX734910	Ran-binding protein 7 / Importin7 (Ipo7) *	AY286403		8E-11	1	Y
CX734865, CX734969	Transferrin (Trf)	AAQ63949		6E-49	2	Y
CX734990	Amine oxidase, flavin containing 1(Aof1)	XP_173173		5E-28	1	Y
CX734943	Copine III (Cpne3)	NP_956461		1E-97	1	
CX734874	**type II CAX cation/proton**	**NM_001025507.**		**3E-18**		

Accession Numbers	Identity[a]	Best match NCBI[b]	Best match MGs[c]	E[d]	F[e]	ERG[f]
	exchanger (cax1)					
6- Cell communication- Signal transduction [GO:0007165]						
CX734858	SPRY domain-containing SOCS box protein (Ssb1)	NP_079382		1E-16	1	
CX734904	Serine/threonine kinase receptor associated protein (Strap)	NP_956598		6E-15	1	Y
CX734935	Protein PM1 (C17orf35)	P17152		6E-09	1	
CX735011	Annexin 6 (Anxa6) *	AY178800		5E-21	1	
CX734981	Phospholipase A2 (Pla2) *	AB050633		2E-20	1	
CX735003, CX735009	Cofilin 1 (Cfl1)	NP_005498		9E-27	2	
7- Cell proliferation [GO:0008283]						
CX734891	Proliferating cell nuclear antigen (Pcna)	AAS67694		4E-25	1	Y
CX734868	Mitochondrial DNA polymerase accessory subunit (Polg2)	AAC51321		2E-50	1	
CX734890	Fibrinogen beta (FgB)	AAA52429		2E-22	1	Y
CX734892	Fibrinogen gamma (FgG)	AAK19752		1E-44	1	
CX735002	Proteoglycan 4 / Megakaryocyte stimulating factor (Prg4)	NP_997918		2E-07	1	
CX734885	Estrogen receptor-binding fragment-associated antigene 9 (Ebag9)	NP_957388		9E-08	1	Y
CX734889, CX734893	RET II oncogene / Golgi autoantigen, golgin subfamilyA member 5 (Golgin-84)	Q8TBA6		4E-09	2	Y
CX734900	Thyroid receptor interacting protein 4 (Trip4)	Q15650		1E-27	1	Y
8- Immune response [GO:0006955]						
CX734936	Complement component C3 (C3)	BAA92285		8E-31	1	Y
9-Energy pathways						
CX734971	Vacuolar ATP synthase subunit H (Atp6v0e)	NP_775377		1E-51	1	
CX735006	Mitochondrial ATP synthase, O subunit (Atp5O)	NP_620238		3E-84	1	
CX734989	ATP synthase mitochondrialF1 complex assembly factor 2 (Atpaf2)	CAG04462		4E-76	1	
CX734976	Aldolase C (Aldoc) *	AF041454		2E-18	1	
CX734929,	NADH dehydrogenase (ubiquinone)	NP_777245		1E-72	1	Y

Accession Numbers	Identity[a]	Best match NCBI[b]	Best match MGs[c]	E[d]	F[e]	ERG[f]
CX734930	Fe-S protein 1 (Ndufs1)					
CX734951	**Cytochrome c oxidase subunit III (MT-CO3)**		**SeabassC-CL8Contig1**	**3E-74**		
10-Structural proteins						
CX734928	Golgi peripheral membrane protein p65 (Gorasp1)	NP_062258		9E-40	1	
CX734855	Lamin B3 (LmB3)	BAB32977		5E-22	1	Y
11- Unknow process						
CX734871	Warm-temperature-acclimation-related-65kDa-protein-like-protein (Wap65-2)	BAD18110		4E-74	1	
CX734879, CX734880	Nuclear RNA helicase, DECD variant of DEAD box family (Ddx39)	NP_998142		1E-84	1	
CX734883	Envelope protein, endogenous retrovirus	AAM34209		7E-02	1	
CX734873	**TBC1 domain family, member 23 (Tbc1d23)**	**XM_001337165**		**8E-44**		
CX734861	**hematological and neurological expressed 1-like (HN1L)**	**BK001569**		**1,00E-15**	1	
CX734949	**smoothelin-like protein (smtnl)**	**DQ465377**		**3,00E-14**		
CX734993	**Protocadherin-like wing polarity protein stan precursor**		**Seabream_2006-01-03-CL4665Contig1**	**1,85E-45**		
CX735015	**nf-kappab inhibitor-like protein 1NFKBIL1**		**cDN02P0004C14.F.ab1**	**9,97E-84**		
CX734965	**sperm associated antigen 17 (Spag17)**	**NM_028892**		**3,00E-06**		

[a]Gene identity determined through Blast searches against NCBI

[b]Best NCBI blastx protein sequence match (lowest E value) or, if no match, best BLASTN.

[c]Best MGE/libraries blastx protein sequence match (lowest E value) or, if no match, best BLASTN

*****Nucleotide sequence match.

[d]E (expected) value

[e]Number of clones sampled

[f] Y - indicates genes previously identified as ERG, based on the ERG database.

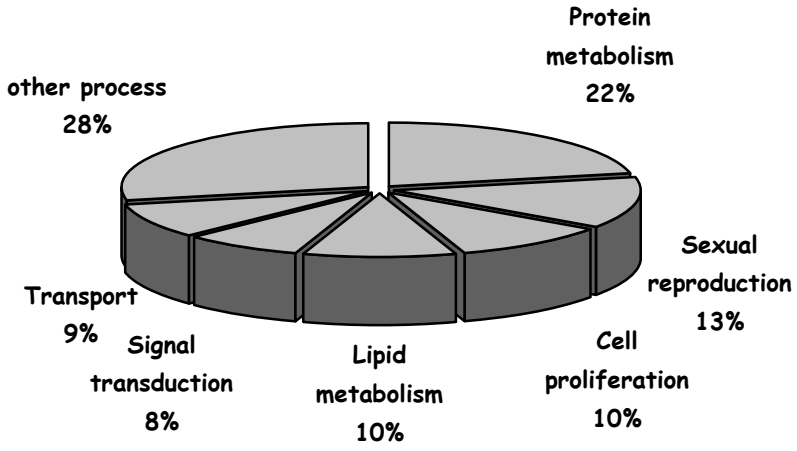

Figure 2 - Distribution of identified genes by biological process categories. Values are the number or percentage (%) , out of the 77 genes for which an ortholog was established.

Table 4 - Distribution of identified genes by biological process categories. Values are the number (Nof genes within each group, out of the 77 genes for which an ortholog was established.

Biological Process	**N. genes before**	**N. genes updated**
Protein metabolism	12	17
Sexual reproduction	9	10
Cell proliferation	8	8
Lipid metabolism	7	8
Signal transduction	6	6
Transport	6	7
Energy pathways	5	6
Nucleic acid metabolism	3	4
Unknown process	3	9
Structural proteins	2	2
Immune response	1	1
	62	77

Values are the number (N) of genes within each group, out of 15 new genes which an ortholog was established in the present analysis.

Discussion

The identity searches in the previous analysis (Pinto et al., 2006b) resulted in the identification of 129 clones, which were particularly enriched of gene products known to be also responsive to E2 in liver (vitellogenin and choriogenins and Apoa I). However 54 clones were not identified and 36 were annotated as unnamed proteins because by that time homologue sequences were not available in the public data bases, however updates of public data bases allowed the identification of 7 clones in the present analysis. However, the identity searches against sea bream and sea bass MGE-EST libraries allowed the identification of 16 clones. This identity search is done in two steps: 1) by homology with longer ESTs annotated in the sea bream and sea bass MGE-libraries; 2) by homology of those ESTs with public data bases. This approach allows to overcome drawbacks like the use of small fragments (average size SSH ~450 Bp) which could be placed in low conserved regions (e.g. un-translated regions), and therefore might not be identified by public data bases search only.

In conclusion, 15 new genes were selected as candidate ERGs, which may be important effectors of estrogen actions. For some of the genes identified in this analysis some functions correlated to development and reproduction has been reported in other organism. For example, HN1L one of the new identified genes is proposed to be involved in embryo development and it is expressed abundantly in human testis and at varying levels in liver, kidney, prostate and uterus (Zhou et al., 2004). Smoothelin protein was also identified in humans, which has been reported as a new cytoskeletal protein which is only found in contractile smooth muscle cells and does not belong to one of the classes of structural proteins presently known (van der Loop et al., 1996) it was also found to these gene be responsive and up-regulated by E2 in cultured human prostatic stromal cells. In *Drosophila melanogaster* SLBP together with U/ has been suggested to cooperate in the production of histone mRNA and that the disruption of histone processing is detrimental to development (Godfrey et al., 2006). Slbp null mutations cause lethality and U7 null mutants develop into sterile males and females, and these females display defects during oogenesis similar to germ line clones of Slbp null cells. Has been suggested that CD9, a member of the tetraspanin family of proteins may control the prostate cancer progression in humans (Zvereff et al., 2007). Also, has been reported that inhibition of phosphoprotein

phosphatase 2A (PPP2CA) in mouse oocyte results in aberrant chromatin condensation and consequently to inability to resolve bivalents (Swain et al., 2007).

The identification of these genes can help to better understand the estrogen functions in male fish physiology. However, differential expression of cDNAs from individuals of control fish and E2-treated should be confirmed by RT-PCR or by qPCR, as previously done by Pinto, et al. (2006b) for some clones. If some of these genes were confirmed to be E2-regulated in sea bream testis it would be interesting to clone the full-length sequences and study their eventual utilization as biomarkers of endocrine disruption.

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REAGENTS

◊ **Solutions used in agarose gel electrophoresis**

6x DNA loading buffer
40 % (w/v) sacarose, 0.25% (w/v) bromophenol blue in stH_2O. Store at 4ºC.

10x TBE (Tris-Borate-EDTA) buffer
108 g of Trizma base
55 g of boric acid
40 ml of 0.5 M EDTA pH 8.0
Add dH_2O to 800 ml, mix until it dissolves, check the pH and if necessary adjust to 8.3 with boric acid. Add dH_2O to 1 L and autoclave.

◊ **Protocol for agarose gel electrophoresis to analyse DNA**

Dissolve agarose in 1x TBE (according the size of fragment to be separated *), melt at 100ºC in a microwave oven and cool to 55ºC before addition of ethidium bromide to a final concentration of 0.5 μg/ml. Pour this solution into a gel cast with combs assembled, allow to solidify at room temperature for 30- 40 min and transfer to an electrophoresis tank filled with 1x TBE buffer.

Choose an appropriate DNA molecular weight marker to estimate the size of fragments in the sample. Markers used in this project were the 1 kb Plus DNA ladder (Invitrogen.

Mix samples or marker with 0.2 volumes of 6x DNA sample buffer load the gel and run at 4-5 V/cm for an appropriate time. Visualize the gel on an UV light transilluminator and photograph.

* -0.7% agarose (w/v) to separate fragments > 2000 bp;
-1% agarose for fragments between 250 and 2000 bp;
-1.5% agarose for fragments < 250 bp.

◊ **Protocol for agarose gel electrophoresis to analyse total RNA**

Prepare 1x TBE buffer by diluting the 10x TBE stock in DEPC water. Use this buffer to prepare a 1% agarose gel as indicated above and fill an electrophoresis tank, previously washed in 1% SDS for 1 h followed by DEPC water. Mix samples with 0.2 volumes of 6x RNA sample buffer, load the gel, run at 4-5V/cm and visualize on an UV light transiluminator.

◊ **Solutions used for SDS-PAGE**

2x SDS sample buffer
100 mM Tris-HCl pH 6.8, 200 mM DDT, 4%(w/v) SDS, 0.2%(w/v) bromophenol blue, 20%(v/v) glycerol.

1.5 M Tris, pH 8.8
Dissolve 36.2 g of Tris-base (Merck) in 150 ml ddH_2O, adjust pH to 8.8 with concentrated HCl and add ddH_2O to 200 ml.

1 M Tris, pH 6.8
Dissolve 12.1 g of Tris-base (Merck) in 75 ml ddH_2O, adjust pH to 6.8 with concentrated HCl and add ddH_2O to 100 ml.

5x Tris-Glycine running buffer
25mM M Tris-HCl, 250mM glycine buffer, 0.1%SDS pH 8.8
Dissolve 15.1g of Tris base (Merck) and 94g of glycine in 900ml ddH_2O. Add 50 ml of 10%(w/v) SDS and ddH_2O to a final volume of 1L.
To make 1x running buffer, dilute 1:5 with ddH_2O.

Stacking and resolving gel for SDS-PAGE

Reagents	**Stacking Gel** (Total volume = 4 mL)	**Resolving Gel** (Total volume = 10 mL)
Polyacrylamide percentage	**5%**	**15%**
ddH_2O	2.92 ml	3.55ml
40%Acrylamide:Bisacrylamide	0.5 ml	3.75ml
Tris-HCl buffer	0.5 ml of 1M Tris-HCl pH6.8	2.5 ml of 1.5M Tris-HCl pH8.8
10% SDS	0.04 ml	0.1ml
10% Ammonium Persulfate (APS)	0.04 ml	0.1ml
TEMED (N,N,N',N'-Tetramethylethylenediamine)	0.004 ml	0.004 ml

For the resolving gel: combine water, acrylamide, Tris-HCl buffer and SDS. Add freshly prepared 10% (w/v) APS and TEMED, mix well and pour solution into the gel sandwich (plates assembled with 0.75-1.5 mm spacers), leaving sufficient space at the top for the stacking gel. Gently overlay with dH_2O and allow to polymerize for 30 min.
Pour off aqueous layer, prepare stacking gel as above and pour into the gel sandwich. Immediately insert comb and allow polymerising for 30 min.

◊ **Solution for Western blot**

Blocking solution
10% (w/v) dried skimmed milk; 0.1% (v/v) Tween-20 in 1x phosphate buffered saline, PBS

DAB substract solution
0.04% (w/v) 3,3-diaminobenzidine (DAB, Sigma), 0.02 %(w/v) nickel chloride, 0.0025%(v/v) hydrogen peroxide in 1x PBS

10x PBS (Phosphate Buffered Saline)
80 g of NaCl (Sigma)
2 g of KCl
14.4 g of Na_2HPO_4
2.4 g of KH_2PO_4
Add ddH_2O to 1 L and adjust pH to 7.4 with concentrated HCl.
To make a 1x PBS dilution, dilute 1:10 with ddH_2O.

Washing solution PBST (PBS-Tween)
PBS 1x with 0.05% (v/v) Tween-20

Western Transfer buffer
Dissolve 3.03g of Tris base (Merck) and 28.8 g of glycine in 800 ml ddH_2O. Add 100 ml of methanol, 5 ml of 20% SDS and ddH_2O to 1 L. Store at 4ºC until use.