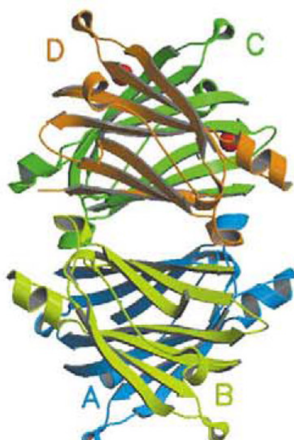
UNIVERSIDADE DO ALGARVE

Faculdade de Ciências do Mar e do Ambiente

Transthyretin and thyroid hormone transport in fish and the effect of endocrine disruptors on this process

(Tese para obtenção do grau de doutor no ramo de Biologia, especialidade de Biologia Molecular)



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Faro
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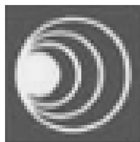

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Cover image*: crystal structure of sea bream Transthyretin. The four subunits that compose the tetramer are labelled A-D and shown in different colours. In Folli et al., 2003*

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During this PhD I have learned about science and its “pathways” and also about life and its lessons. I found out that I didn’t have a clue about both…but now I’m in a better position to get some. For such big accomplishment I have to acknowledge many people:

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NOME: Isabel Maria Sena Morgado

FACULDADE: Ciências do Mar e do Ambiente

ORIENTADOR: Professora Deborah Power

DATA: Março 2007

TÍTULO DA TESE: Transtirretina e o transporte das hormonas tiróides em peixes e o efeito dos disruptores endócrinos neste processo

RESUMO

Nos vertebrados, a transtirretina (TTR) é uma proteína responsável pelo transporte sanguíneo das hormonas tiróides (HT), tiroxina (T_4) e triiodotironina (T_3). A evolução estrutural e funcional da TTR nos vertebrados, a sua capacidade de formar fibras amilóides causando doenças humanas e a sua afinidade para disruptores endócrinos (DEs) são questões importantes pouco exploradas em peixes. Neste estudo produziu-se TTR recombinante de dourada (sbrTTR), antisoro específico anti-sbrTTR e dois mutantes sbrTTR no N-terminal. Ensaios competitivos de ligação demonstraram que sbrTTR liga-se com afinidade semelhante à T_3 (Kd=10.6nM) e T_4 (Kd=9.8nM) e que o N-terminal influencia a ligação à sbrTTR e a produção de fibras amilóides. Níveis plasmáticos de TTR (3.8µg/ml) aumentaram após administração de HT apesar da expressão génica hepática não ser alterada, sugerindo que as HT regulam a secreção da TTR mas não a síntese. Os DEs competiram significativamente na ligação sbrTTR-HT *in vitro*, indicando a utilidade deste ensaio na avaliação da disrupção do eixo da tiróide em peixes. *In vivo*, os ligandos da TTR, ioxynil e diethylstilbestrol, alteram o eixo da tiróide em dourada; a expressão de TSH e deiodinases no cérebro diminuiu apesar dos níveis plasmáticos das HT e a expressão hepática da TTR não sofrerem quaisquer alterações.

Palavras chave: Transtirretina, proteínas de ligação às hormonas tiróides, hormonas tiróides, peixes teleósteos, sistema da tiróide, disruptores endócrinos.

THESIS TITLE: Transthyretin and thyroid hormone transport in fish and the effect of endocrine disruptors on this process

ABSTRACT

Transthyretin (TTR) is a homotetrameric protein that transports thyroid hormones (THs) thyroxine (T_4) and triiodothyronine (T_3) in vertebrate's blood. TTR features raise important questions: understanding its structural/functional evolution in vertebrates; its ability to form amyloid fibrils causing human disease and TTR's high affinity for endocrine disruptor chemicals (EDCs). In fish, TTR features are poorly explored. In this study, sea bream recombinant TTR (sbrTTR), specific anti-sbrTTR antisera and two sbrTTR N-terminal mutants were produced. A [^{125}I-T_3] competitive binding assay demonstrated that sbrTTR binds T_3 (Kd=10.6nM) and T_4 (Kd=9.8nM) with a similar affinity and the N-terminus was found to influence binding to sbrTTR but also the formation of fibrils. TTR plasma levels, measured (3.8μg/ml) for the first time in fish using a specific ELISA, increased upon administration of THs, although hepatic gene expression was not modified suggesting THs regulate TTR secretion but not synthesis. Putative EDCs strongly displace *in vitro* sbrTTR-THs binding indicating the sbrTTR binding assay could be a useful risk assessment tool for thyroid axis disruption in fish. *In vivo*, TTR-binders ioxynil and diethylstilbestrol altered the thyroid axis in sea bream; brain TSH and deiodinases expression was downregulated although TH plasma levels and TTR hepatic expression remained unaltered.

Key-words: Transthyretin, thyroid hormone binding proteins, thyroid hormones, teleost fish, thyroid system, endocrine disrupting chemicals.

List of Publications and Communications

Articles in refereed journals

Morgado, I., Santos, C.R.A., Jacinto, R., Power, D. M. 2007. Regulation of Transthyretin by thyroid hormones in fish. Gen Comp Endocrinol. 152, 189-197.

Morgado, I., Hamers, T., Van der Ven, L., Power, D.M. 2007. Disruption of thyroid hormone binding to sea bream recombinant Transthyretin by ioxynil and polybrominated diphenyl ethers. Chemosphere. 69, 155-163.

Conference proceedings

Morgado, I., Anjos, L., Power, D. M. 2005. Transthyretin and thyroid hormone transport in fish. Avances en Endocrinología Comparada vol.II, 277-79.

Morgado I., Sauer-Eriksson, E., Power, D. M. 2006. Thyroid hormone binding by recombinant sea bream transthyretin: The role of the N-terminal region Journal of experimental zoology. Part A- comparative experimental biology 305A (2): 158-158 FEB 1 2006

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18S	18S ribosomal RNA
ALB	Albumin
ANOVA	Analysis of variance
BDE	Brominated diphenyl ethers
BFR	Brominated flame retardants
bp	Base pairs
BPG axis	Brain-pituitary-gonads axis
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CD	Circular dichroism
cDNA	Complementary DNA
cGMP	Cyclic guanosine monophosphate
CNSA	Central nervous system selective amyloidosis
CSF	Cerebrospinal fluid
DAB	3,3-diaminobenzidine hydrochloride
DAG	Diacylglycerol
DBD	DNA-binding domain
DEPC	Diethylpyrocarbonate
DES	Diethylstilbestrol
DIT	Diiodotyrosine
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleotide triphosphate
DTT	Dithiothreitol
ECL	Enhanced Chemiluminescense
EDC	Endocrine disrupting chemicals
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FAC	Familial amyloid cardiomyopathy
FAP	Familial amyloid polyneuropathy
HSA	Human serum albumin
HBP	Halogen binding pockets

HPLC	High performance liquid chromatography
IP_3	Inositol triphosphate
IPTG	Isopropyl-beta-D-thiogalactopyranoside
IRD	Inner-ring deiodination
Kb	Kilo bases
***K*d**	Dissociation constant
kDa	Kilo Dalton
MCS	Multiple cloning site
MIT	Monoiodeotyrosine
MMI	Methimazol
MMLV-RT	Mouse Moloney murine leukemia virus reverse transcriptase
MOPS	3-(N-Morpholino) propanesulfonic acid
mRNA	Messenger ribonucleic acid
MW	Molecular weight
OD	Optical density
ORD	Outer-ring deiodination
PBDE	Polybrominated diphenyl ethers
PCB	Polychlorinated biphenyls
PCR	Polymerase chain reaction
PEG	Polyethilene glycol
PFA	Paraformaldehyde
PMSF	Phenylmethylsulphonyl fluoride
Poly(A)	Polyadenylated RNA (mRNA)
PTU	Propilthyouracil
PVDF	Polyvinylidene difluoride
RBP	Retinol-binding protein
RIA	Radioimmunoassay
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Rotations per minute
RT-PCR	Reverse transcriptase-polymerase chain reaction
RXR	Retinoid X receptor
sbrTTR	Sea bream recombinant TTR
sbrTTRM6	Sea bream recombinant TTR lacking amino acids 1-6
sbrTTRM12	Sea bream recombinant TTR lacking amino acids 1-12

sbrTTRWT	Sea bream recombinant TTR wild type
sbTTR	Sea bream TTR
SDS-PAGE	Sodium dodecyl sulphate - polyacrylamide gel electrophoresis
SE	Standard error
SSA	Senile systemic amyloidosis
T_3	Triiodothyronine
T_4	Thyroxine
TBBPA	Tetrabromobisphenol A
TBG	Thyroxine-binding globulin
TBPA	Thyroxine-binding prealbumin
TG	Thyroglobulin
TH	Thyroid hormones
THBP	Thyroid hormone binding protein
ThT	Thioflavine T
TPO	Thyroid peroxidase
TR	Thyroid hormone receptors
TRE	Thyroid hormone response elements
TRH	Thyrotropin-releasing-hormone
TSH	Thyrotropin-stimulating-hormone
TTR	Transthyretin
U	Units
X-Gal	5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside

General Introduction

1.1 *Thesis context*

The present manuscript describes research work developed within the aim of the thesis to give an overview of the thyroid hormone axis and the thyroid hormone binding proteins. It is focused on a quite specific aspect of the vast field of Biology, although many tools and sub-fields of this multidisciplinary area were obviously involved. The research field addressed by the thesis and in particular the introduction would fall within the realms of Fish Thyroidology. This, in its turn, can be included in Comparative Endocrinology, a branch of Endocrinology, and an important sub-discipline of Animal Physiology.

Humankind has been dealing with aspects of endocrine physiology and pathology for thousands of years (e.g., domestic castration of dogs to modify behaviour). For instance, description of dysfunctional thyroid gland symptoms and respective treatment with the iodide rich sea water date back over 3.500 years (Medvei, 1982). However, the concepts of “endocrine” and “hormone” were only established at the beginning of the twentieth century (Bayliss and Starling, 1901/1902; Bayliss and Starling, 1902) and since then the field of Endocrinology has been evolving rapidly. The progress in science and technology allowed hormone identification, characterization and provided means of quantification. It also permitted great advances in the understanding of endocrine glands secretory mechanisms, hormone and receptor structures and interactions and signal transduction. Most of these achievements however, have been mainly focused on mammalian systems and in particular the human endocrine system due to its interest for human medicine. Studies focused on non-mammalian animals have been grouped together and classified as “Comparative Endocrinology”. This new field was initially based upon comparisons of “lower-vertebrates” with the better known mammalian model. However, the limited value of such extrapolations is clear when considered in

the context of the entirely different physiological challenges faced by mammals and lower vertebrates in their environments. Today, the concept of comparative endocrinology mostly relies in the search of "commonalities in the endocrine physiology of different vertebrate and invertebrate organisms with a view to establishing underlying principles and evolutionary relationships of the groups" (Leatherland, 1993).

Over the past 20 years Comparative Endocrinology started to devote increasing attention to a specific taxon such as fish in which the endocrine system regulates many fundamental processes (e.g. growth, development, metabolism and reproduction). This phenomenon is also probably explained by the key position of fish in evolution and the fact that they represent the most abundant vertebrates on the planet (over 25,000, Helfman et al.(1997)). Moreover, the perceived health benefits of consuming fish have made them of increasing commercial importance as a food resource, but there is also a buoyant sport fishing and aquarium hobbyist industry.

The present work is focused on a very specific aspect of fish endocrinology or more precisely fish thyroidology: thyroid hormone transport. All the studies herein described concern Transthyretin (TTR), one of several thyroid hormone binding proteins which have been described and characterised in vertebrates. Human TTR is especially well characterised and is an important subject of study due to its role in severe neurodegenerative diseases (e.g. familial amyloidotic polyneurpathy (FAP), senile systemic amyloidosis (SSA)). In fish, however, relatively little is known about this binding protein. Only recently TTR was identified in teleost fish (Funkenstein, 2001; Kawakami et al., 2006; Santos and Power, 1999; Yamauchi et al., 1999). When the present thesis project was conceived and planned, studies concerning fish TTR were very scarce or almost inexistent although there was evidence (mostly from other

vertebrates) of many interesting aspects to explore: TTR binding to thyroid hormones (THs) and the molecular basis of this process; its role in the thyroid axis and involvement in TH regulation; TTR misfolding and stability a causative factor in human diseases and also the ability of TTR to bind endocrine disrupting chemicals (EDC). Furthermore, the sequence of TTR from a teleost fish, the sea bream, had recently been cloned and preliminary studies of its structure were ongoing which made it the perfect tool. In light of the scarcity of information about fish TTR and taking into consideration its potential importance in a number of important aspects of vertebrate thyroidology the present project was undertaken. In the present thesis a number of tools with which to study fish TTR were developed and applied in structural, biochemical and physiological studies. They have responded to some questions about fish TTR but mostly they have raised many new interesting ones about this proteins evolution and function.

1.2 *The Endocrine system*

As life evolved in its complexity, multicellularity and differentiation gave rise to the extremely organised forms of life presently found in nature. Such multicellular organisms are in the end no more than an orchestra of cells performing different functions as an integrated whole and for this reason coordination is a key factor. Efficient organization and coordination requires an effective communication network and cells in close connection communicate directly by electrical and chemical interactions. However, as animals became larger networks were required to sense and bring about rapid responses to specific stimulus, two important networks which evolved were the nervous and the endocrine system. In the latter system, information is integrated in a set of chemical messengers known as “hormones” (from the Greek hormao = to excite) which are produced by a number of endocrine glands widely spread

throughout the body. In general, endocrine integration controls long-term processes like growth, development, metabolism, reproduction, etc.

The term hormone was introduced for the first time by Bayliss and Starling in 1902. They demonstrated unequivocally, when trying to identify the factors responsible for pancreatic secretion, that this vital process is regulated by a hormone, secretin, and defined in their study the concept of "endocrine". The definition of an endocrine hormone is a "*factor secreted from a gland into the blood stream which acts at distant targets*". However, with the advance of the field of endocrinology a number of new concepts have been introduced. Currently, it is established that hormones may circulate in the blood, other body fluids or by diffusion between cells and their action can be endocrine (if the target is a distant organ) but also autocrine (when acting on the same cell type that secreted them or paracrine (if acting on neighbouring cells of different type). In 1989, (Bolander, 1989) defined a hormone as a "chemical, non-nutrient, intercellular messenger carrying information between two or more cells".

Different chemical compounds can act as hormones. They can be peptides and proteins, steroids or amino acid derivatives. The structural and chemical properties of each hormone greatly determine their mode of action. These chemical messengers are synthesized by the metabolic machinery of the gland cells, stored in vesicles or granules and released, on appropriate stimulation, by exocytosis directly into the blood or other body fluids. The stimulus for release is usually a consequence of alterations in the external environment (e.g. temperature) or *in vivo* (e.g. nutritional status) and can be transmitted as an electrical or chemical signal like a neurotransmitter, nutrient or other hormone. Once in the circulation (or in the extracellular space) the released hormone travels to its effector sites. Most water-soluble hormones, like peptides, are carried in physical solution while hydrophobic and lipid soluble hormones (like steroid or some

peptide derivatives) are carried bound to plasma proteins. When reaching the target cell the hormone is recognized by specific receptors in the plasma membrane, cytoplasm or nucleus. Hormone-receptor binding gives rise to a cascade of intracellular events that ultimately lead to the hormone´s physiological effect (e.g., changes in enzyme activity, ion levels, transcription of responsive genes, etc). In general, small lipophilic hormones like steroid or thyroid hormones pass through the lipid plasma membrane of receptor cells and bind a cytoplasmic or nuclear receptor. The complex formed binds to acceptor sites on the chromosome regulating the transcription of specific genes, the products of which are usually enzymes. In contrast, peptide or protein hormones which are water-soluble do not cross the plasma membrane and act by binding to transmembrane receptors on the cell surface. The hormone-receptor complex activates an enzyme inside the membrane, which in turn leads to the formation of a second intracellular messenger (e.g. cyclic adenosine monophosphate (cAMP), cyclic guanosine monophosphate (cGMP), diacylglycerol (DAG), inositol triphosphate (IP_3) and Ca^{2+} (Bentley, 1998; Lodish et al., 2000). The secondary messengers trigger a cascade of intracellular responses, generally involving activation of ion channels or enzymes such as protein kinases that give rise to the hormone's physiological effect. The response is of a rapid non-genomic kind unlike that generally provoked by hormones binding to nuclear receptors. However, recently membrane receptors have also been shown to control transcription of specific genes through transcription factor activation (Lodish et al., 2000; Neves et al., 2002) and nuclear receptors located in the cytoplasm and cell membrane appear to interact with intracellular signalling factors mediating non-genomic actions (Aranda and Pascual, 2001).

Hormone concentrations in the blood stream are usually very low but cause profound effects as a consequence of their amplification by the cascade of events they activate.

However the triggering of such events is highly dependent on circulating hormone concentrations which are mainly determined by their rate of secretion and clearance. The balance of circulating hormone levels is crucial and is generally regulated by a feedback system. In this way, negative feedback loops, often involving two or more glands, act inducing a gland to raise/decrease hormone secretion in response to lower or higher circulating hormone concentrations. One well established example of this mechanism is the hypothalamus – pituitary - thyroid axis which will now be described as it provides the background for the present thesis.

1.3 Thyroid axis and hormones

The presence of a thyroid gland in mammals has been recognised for several thousand years (Leatherland, 1993); in humans it consists of a small bilobed structure that develops embryonically as an outgrowth from the front of the pharynx and migrates in a posterior direction to the ventral surface of the neck surrounding the trachea (Dorit et al., 1991). This position is generally comparable in all vertebrates. Structurally, the thyroid gland consists of a follicle formed by a single layer of epithelial cells surrounding a central cavity filled with colloid. In an active thyroid gland the epithelial cells of the follicle are columnar and the colloid contains numerous vacuoles while in an inactive gland the epithelial cells are flattened and follicles are distended with colloid (Bentley, 1998). The thyroid appears to have the longest phylogenetic history of any endocrine gland and its basic follicular unit has been conserved in all vertebrates and homologous tissues have been identified in protochordates, including amphioxus (Cephalochordata) and ascidians (Urochordata) (Barrington, 1962).

The thyroid gland exerts its action through the synthesis of two biologically active hormones, Thyroxine (T_4) and Triiodothyronine (T_3) the production of which is

regulated by a feedback inhibition mechanism operating at the level of the hypothalamus - pituitary gland - thyroid gland axis. Thyroid follicles have the remarkable ability to extract iodine from the blood which is essential for thyroid hormone synthesis. Iodine uptake is a critical step for hormone production and by regulating this step the thyroid gland regulates its own activity. Thyroid hormone levels in the blood are primarily controlled by negative feedback at the hypothalamus (fig.1). This system-wide regulatory centre located in the brain, synthesizes and releases thyrotropin-releasing-hormone (TRH) which in turn stimulates the secretion of thyrotropin-stimulating-hormone (TSH) by the pituitary gland. TSH is secreted into the blood by the pituitary gland and acts on the thyroid stimulating the synthesis and release of biologically active TH molecules. Synthesis of TRH by the hypothalamus is regulated by both the concentration of THs and TSH in the blood. A reduction in the concentration of circulating THs is also directly sensed by the pituitary thyrotrophs and stimulates TSH release.

1.3.1 *Thyroid hormones*

Thyroid hormones have been identified throughout the vertebrates including basal groups such as, cyclostomes, teleosts, amphibians and reptiles (Chiu et al., 1975; Higgs and Eales, 1973; Packard et al., 1976) and iodothyronine active compounds were also found to be present in a number of protochordates (Bentley, 1998). The thyroid hormones are probably the most pluripotent hormones found in vertebrates as they can influence virtually every metabolic activity of an organism (e.g., concentration and function of numerous enzymes; metabolism of fats, carbohydrate, vitamins, proteins; heart rate; stimulation of erythropoiesis or regulation of bone synthesis, etc.).

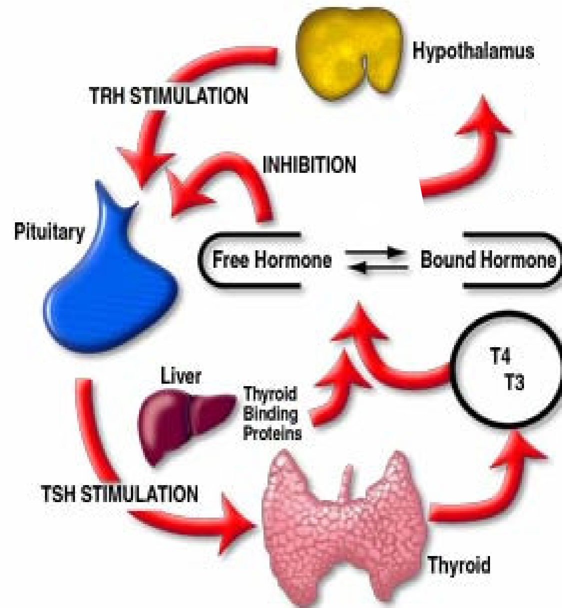

Figure 1 Hypothalamic - pituitary gland - thyroid gland axis. Thyroid hormone levels are regulated by a feedback inhibition mechanism which operates along the hypothalamic-pituitary-thyroid axis. The hypothalamus secretes thyrotropin releasing hormone (TRH) which stimulates the pituitary to secrete thyroid stimulating hormone (TSH). TSH, in turn, stimulates the thyroid gland to produce and secrete thyroid hormones (T_4 and T_3) into the circulation where they bind thyroid binding proteins. The system attempts to maintain constant the free TH fraction through a negative feedback loop. Alterations on free hormone concentrations are sensed at the level of the hypothalamus and pituitary and TSH production is stimulated/inhibited in order to re-establish adequate TH levels.
Adapted from: http://www.dpcweb.com/medical/thyroid/thyroid_function.html

Physiologically, THs act through two major mechanisms: by increasing protein synthesis and oxygen consumption. Ultimately, their action influences basic life cycle events such as growth, differentiation, metamorphosis, reproduction, hibernation and thermogenesis (Bentley, 1998). Normal thyroid function is essential for both mental and physical development of young mammals. Inadequate production or overproduction of thyroid hormones is strongly associated with two types of thyroid disease states in humans, hypothyroidism and hyperthyroidism, respectively. In children, low levels of

THHs (hypothyroidism) can compromise normal physical and mental development, a conditions known as cretinism. The same condition in adults results in low metabolism and several associated complication like weight increase, low body temperatures, etc (Dorit et al., 1991). Usually hypothyroidism can be readily treated by oral administration of synthetic THs. Abnormally high TH levels (hyperthyroidism) causes an elevation of the metabolic rate, weight loss, excessive perspiration, nervousness and other complications (Dorit et al., 1991) Hyperthyroidism is commonly treated by administering anti-thyroid drugs (e.g. propylthiourea, methimazole), which suppress synthesis of thyroid hormones primarily by interfering with iodination of thyroglobulin.

In other vertebrates the function of THs is still not fully understood. For example they are essential for moulting in amphibians and reptiles, and their essential role in amphibian methamorphosis is widely recognised (Leloup and Buscaglia, 1977). Recent reports also highlight their importance in fish development, growth and metamorphosis (Power et al., 2001; Yamano, 2005). In fact, many vertebrates have a characteristic rise in THs during crucial stages of development (Hulbert, 2000; Leloup and Buscaglia, 1977).

Functional THs are small amino acid derivatives (fig. 2) consisting of a hydrophobic thyronine nucleus, which accounts for their poor water solubility, a hydrophilic hydroxyl group attached to the phenolic ring and four or three iodines at positions 3, 5, 3’, and 5’ in thyroxine (**T_4**) or 3, 5, 3’ in triiodo L-thyronine (**T_3**) (Power et al., 2000a).

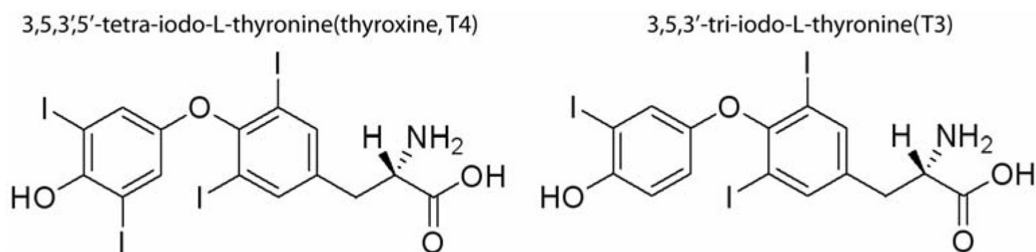

Figure 2 The chemical structure of thyroid hormones T_4 (left) and T_3 (right) (Lundberg, 2006)

1.3.2 *THs biosynthesis*

The general principals of thyroid hormonogenesis as presently accepted were first outlined by Gorbman and Bern (1962) and are essentially similar throughout vertebrates (Leatherland, 1993). TH synthesis takes place in the follicular cells of the thyroid and starts with the uptake of extracellular iodide. See fig. 3 for a schematic representation of TH biosynthesis. The thyroid utilizes I^-, the elemental form in the blood, which is ultimately derived from the environment. Terrestrial vertebrates obtain I^- mostly from food while fish take up iodine from the water via the gills and gut. Iodide is pumped into thyrocytes in an ATP dependent manner by means of a sodium/iodide co-tranporter (symporter, NIS) protein in the plasma membrane. Once inside the follicular cell it is converted by thyroid peroxidase (TPO), most likely at the luminal surface of the cell, to an oxidized species of iodine which is incorporated into tyrosyl groups of thyroglobulin (TG) a very large glycoprotein. Iodination occurs at specific tyrosine sites within thyroglobulin giving rise to diiodotyrosine (DIT) and monoiodeotyrosine (MIT). TPO activity facilitates the intramolecular coupling of either two DIT residues, giving rise to tetraiodothyronine (T_4), or a MIT with a DIT residue resulting in the formation of

triiodothyronine (T_3). At this stage, iodinated thyronine compounds are an integral part of thyroglobulin and represent a reserve of THs stored in the lumen of the thyroid follicles. Enzymatic degradation of the thyroglobulin, stimulated by TSH, releases THs into the intracellular compartment. Free T_4 and smaller amounts of free T_3 are then secreted into the blood. While T_4 is formed exclusively in the thyroid, 80% of T_3 found in circulation is thought to derive from T_4 conversion in peripheral non-thyroidal tissues (especially liver and kidney). The difference between the source of T_3 and T_4 is probably related to their mode of action. T_4 is considered to be a prohormone as in order to act it has to be converted to its derivative, T_3, the biologically more potent TH because of its greater affinity for thyroid hormone receptors (TRs) (McNabb, 1992).

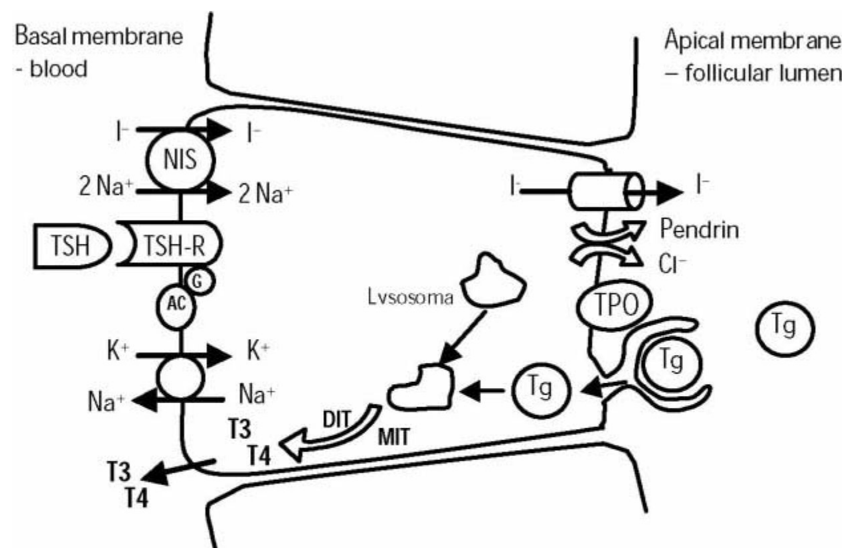

Figure 3. Thyroid hormones biosynthesis pathway in a thyroid follicle cell. The first step to hormone synthesis is the import of iodide into the follicular cells, an active transport process (an ATP-pump pumps K^+ in and Na^+ out of the cell) that occurs through a sodium/iodide "symporter" (transporting iodide in). Once in the follicular cell, iodide is converted into iodine by the enzyme thyroid peroxidase (TPO), which uses hydrogen peroxide (H_2O_2) as a cofactor. Thyroid peroxidase catalyzes the incorporation of iodide molecule onto both the 3 and/or 5 positions of the phenol rings of tyrosines present in the large protein thyroglobulin (TG) originating diiodotyrosine (DIT) and monoiodeotyrosine (MIT). Thyroglobulin contains 140 tyrosines but only two to five of these are converted into either T_4 or T_3 through TPO activity. TSH stimulates enzymatic degradation of thyroglobulin releasing the THs into the intracellular compartment which are further secreted into the blood plasma. Original picture from Boas, et al (2006).

1.3.3 *TH mode of action and metabolism*

Once released into the circulation, a major part of THs reversibly binds to plasma transport proteins and only a minor fraction (approximately 0.03% for T_4 and 0.3% for T_3) remains in the free form (fig.1). According to the "free hormone hypothesis" formulated by Robbins and Rall in 1960 and further supported by Mendel and colleagues (Mendel, 1989), thyroid hormones enter target cells by diffusion and it is the free hormone and not the protein-bound one that is available to enter cells. Therefore, the free extracellular TH concentration constitutes the driving force that determines the rate at which THs reach their targets eliciting physiological responses. However, an alternative proposal (Beraud et al., 1958 and Ingbar and Freinkel, 1960) suggests that transmembrane diffusion of THs is governed by intra and extra-cellular thyroid-hormone binding proteins. More recently several studies have demonstrated the existence of a cellular uptake mechanism for THs in which binding proteins mediate cell entry through protein receptors on the membrane of the target cell (Benvenga and Robbins, 1990; Divino and Schussler, 1990; Kuchler-Bopp et al., 1998; Vieira et al., 1995). Although this membrane translocation mechanism is not completely clarified it is presently accepted that cellular uptake of THs is a carrier-mediated process important for overall regulation of their bioactivity (for review see Hennemann et al., 2001).

TH actions after cellular uptake are mediated by nuclear thyroid hormone receptors (TRs) that can bind T_3 with high affinity (for reviews see Wu and Koenig, 2000; Zhang and Lazar, 2000). TRs belong to the nuclear receptor superfamily that also includes receptors for other small lipophylic hormones (e.g. steroids, retinoids and vitamin D). Two distinct genes (TRα and TRβ) located in different chromosomes (17 and 3, respectively in humans) generate multiple TRs isoforms including TRα1, TRα2, TRα3

and TRβ1, TRβ2 (Lazar, 1993) which have distinct functional roles. TRs act as transcription factors regulating gene expression. They bind to specific regulatory DNA sequences of the target genes known as TH response elements (TREs). Although TR can bind TREs as monomers or homodimers, they preferentially bind many TREs as heterodimers with the retinoid X receptor (RXR), another member of the nuclear receptor superfamily. Binding of unliganded TR generally represses basal transcription while ligand (T_3) binding triggers a conformational change in the TR activating transcription of a target gene. Inhibition and enhancement of gene expression can depend on the nature of the TREs, hormonal status and the cellular environment.

TRs like other nuclear receptors exhibit a modular structure with a variable N-terminal region, a central DNA-binding domain containing two zinc fingers and a large highly conserved ligand-dependent activation domain located towards the C-terminus of the protein (Lazar, 1993). The intracellular T_3 concentration largely determines the degree of occupancy of nuclear receptors thus regulating biological responses to THs (Brent, 1994). The intracellular T_3 concentration in its turn is directly influenced by factors such as, T_3 and T_4 uptake rates from the extracellular fluid or TH inactivation. The latter can be achieved by glucuronide or sulphate conjugation, deamination or decarboxylation, but the major metabolic pathway is deiodination which takes place mainly in liver and kidney. T_4 can lose iodine atoms in a stepwise manner to create an array of iodothyronines. The most important deiodination pathway is T_4 conversion to T_3 which occurs by removal of one iodide unit from the outer ring of T_4 (5'-monodeiodination or outer-ring deiodination, ORD). Monodeiodination of the inner ring (5-monodeiodination or inner-ring deiodination, IRD) can also take place giving rise to 3,3',5'-T_3 (reverse T_3 or rT_3). Although, rT_3 has no biological activity this alternative pathway allows control of peripheral hormone levels by converting excess T_4 into non-

active rT_3 which can be further deiodinated to T_2, T_1 and thyronine (T_0). Elevated plasma T_3 levels can also reduce 5'-monodeiodination activity (MacLatchy, 1993; Scott-Thomas et al., 1992) suggesting an autoregulation of T_3 production. This dual control mechanism allows independent regulation of the prohormone T_4 and of the biologically active T_3 blood levels (Leatherland, 1993). Deiodination is carried out by a family of selenoprotein enzymes, the deiodinases. At least three different deiodinases are known in homeotherms, type I (DI), type II (DII) and type III (DIII) (Berry et al., 1991; Croteau et al., 1996; Croteau et al., 1995), which vary in tissue location, substrate preference and affinity (fig. 4B). DI, which can deiodinate both inner and outer ring is commonly found in the liver, kidney, muscle, pituitary and thyroid gland. DII can only deiodinate the outer ring and being the major activating enzyme. It is mostly present in the brain but also in the testis, thyroid, muscle, heart and pituitary. DIII can only deoidinate catalyze inner ring deoidination being the major inactivating enzyme. It is found in the brain and skin and also the placenta and fetal tissue.

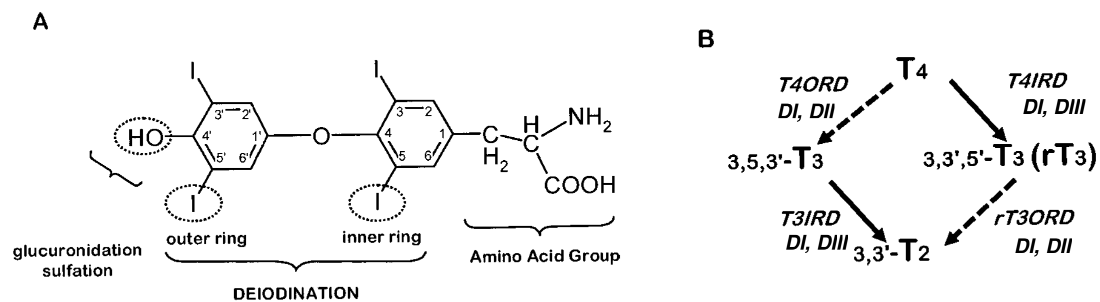

Figure 4. **A**- Structure of L-thyroxine indicating the main pathways of metabolism. Deiodination of T_4 occurs through removal of one iodide unit form the inner (left) or outer (right) ring of the molecule. **B**- Representation of the main deiodination pathways: Outer-ring deiodination (ORD, ---►) and inner-ring deiodination (IRD, →). Active T_3 is formed from T_4 outer ring deiodination (T_4ORD) through the action of deiodinases DI or DII. Inner-ring deiodination of T_4 (T_4IRD) by DI or DIII results in the formation of the inactive reverse T_3 (rT_3). T_3 itself can be degraded by removal of one of a inner (T_3IRD) by DI or DIII or outer-ring iodine (T_3ORD) by DI or DII originating the presumed inactive 3, 3'-T_2. Adapted from Brown et al (2004).

These enzymes act as a complex and coordinated system which enables hormonal activation or inactivation\degradation to playing an important role in modulating TH levels in specific tissues and overall THs homeostasis and economy.

1.3.4 *Thyroid axis in fish*

In higher vertebrates THs have a widespread and diverse action and are essential from early stages of life. Since in lower vertebrates the best known feature of THs is its crucial role in amphibian metamorphosis (Shi et al., 1996), THs are expected to greatly influence embryonic and larval stages of development. In fact, due to its pluripotent action, fish thyroid systems inevitably attract the interest of many fields like fish physiology, biochemistry or aquaculture. The TH axis has also become of considerable concern in environmental toxicology as a number of toxic chemicals may strongly interfere with normal thyroid function, mostly due to their structural resemblance to THs. Throughout the past two decades knowledge about the role of THs in fish has improved and reports mostly relate to their role in regulation of development, growth and aspects of reproduction (for review see Leatherland, 1994; Power et al., 2001; Yamano, 2005) while regulation of basal metabolism, so evident in endotherms, is not so well documented in teleosts.

The existence of a thyroid gland in mammals has been accepted for thousands of years but the recognition of a comparable tissue in fishes only occurred in the mid-late 19th century (Maurer, 1886; Simon, 1844). Unlike tetrapods, which possess a single discrete gland, in fish, thyroid follicles may not be encapsulated by connective tissue. Most orders of teleost fish lack a “glandular” structure and instead thyroid follicles are found scattered throughout the connective tissue of the lower jaw (pharyngeal region) usually aggregated around the ventral aorta (Bentley, 1998; Leatherland, 1994). The essential

components making up the thyroid axis have, however, been largely conserved across vertebrates as has the basic structure and function of fish thyroid tissue. Nevertheless, many aspects of the teleost thyroid axis certainly meet fish physiological adaptations with particular mechanisms of action, some of which are still not completely clarified.

The basic steps involved in fish thyroid function from TH biosynthesis to TH action seem to fit the current model. TSH stimulates secretion from the thyroid which secretes mainly T_4 which is converted in the periphery to T_3 by deiodination. TH transport to target cells involves carrier proteins and T_3 is the active TH form that predominantly binds TRs.

TH biosynthesis depends on an adequate supply of iodide and fish have the capacity to take up iodide from water across the extensive gill surface (Hunn and Fromm, 1966). In teleosts an adequate plasma iodide level is partly determined by dietary and branchial uptake suggesting different strategies in iodide economy. Owing to the greater iodide availability teleosts may have less efficient mechanisms of recovery and retention of hormonal iodide than homeotherms. TH biosynthesis, thyroglobulin properties and intrathyroidal secretion have received limited attention in teleosts. The few studies which exist report that negligible amounts of T_3 (Eales and Brown, 1993) are synthesized or secreted by thyrocytes in teleost species. Although the TH biosynthesis in fish is assumed to fit the mammalian model there are still many gaps in knowledge to be filled. Regulation of thyroid function seems to occur through the hypothalamus-pituitary-thyroid axis, although the identities of the hypothalamic substances influencing teleost thyrotropes are still not known. However, in contrasts to the situation in mammals, T_3 does not appear to influence the activity of the teleost hypothalamus-pituitary-thyroid axis (Eales and Brown, 1993). This fact suggests a loose linkage between the control of T_4 and T_3 metabolism pointing to fundamental differences in

control of thyroid status in teleosts. In contrast to mammals, where plasma T_4 largely exceeds T_3 levels (50-100:1), plasma T_3 levels in teleosts may exceed that of T_4, suggesting a strong 5'-monodeiodination activity (T_4-ORD) and peripheral deiodination seems to be the primary control of teleost thyroid function (Eales and Brown, 1993; Power et al., 2001). It is the regulated conversion of T_4 to T_3 in tissues which may largely determine the T_4 secretion rate from the thyroid. In homeotherms primary control of T_4 and T_3 is largely central through the hypothalamic-pituitary axis. As THs are involved in regulation of basal metabolism rates in homeotherms, this mechanism may have arisen as an advantageous adaptation leading to central control by the hypothalamus, the brain centre associated with thermoregulation.

TH degradation seems to occur mainly through deiodination pathways (Eales and Brown, 1993) but oxidative deamination and decarboxylation also take place. The liver is the pivotal organ in T_4 metabolism and is mainly involved in deiodination and it is the site where TH conjugation and biliary excretion also occur (Power et al., 2001). T_3 is mainly degraded by T_3 IRD in brain, liver and possibly other tissues (Eales et al., 1993b; MacLatchy, 1993). Excretion of TH metabolites was found to occur through the urine and the bile (Eales et al., 1971; Sinclair and Eales, 1972) and other possible routes may be gills, gut or skin.

As in mammals three deiodinase isotypes are expressed in piscine species including agnathans, chondrichthyes and teleosts (Orozco and Valverde-R, 2005). Fish deiodinases share properties with their corresponding counterparts in higher vertebrates. However in fish, these enzymes exhibit distinct features and their physiological role in functions regulated by THs is not completely clear.

Putative nuclear T_3-binding receptors have been described in teleosts in a number of different tissues (e.g., liver, gills, brain, kidney, pituitary and ovary) (Eales and Brown,

1993; Power et al., 2001). Only relatively recently, cloning and characterisation of TRs in fish was carried out (Power et al., 2001; Yamano et al., 1994; Yamano and Inui, 1995) and revealed two principal receptor types, TRα and TRβ. The properties, binding profile and molecular structure of such receptors closely resemble those described in high vertebrates. Therefore they can also be expected to function as transcription factors probably binding similar response elements (Power et al., 2001).

In common with mammals, in teleosts more than 99% of THs do not circulate in the free form (Eales and Shostak, 1985; Weirich et al., 1987) but are covalently bound to plasma proteins (Falkner and Eales, 1973) including lipoproteins (Babin, 1992). In fish thyroid hormone transport by plasma proteins is poorly explored but evidence points to a different general profile. Only two of the three thyroid hormone binding proteins (THBP) present in the blood of larger mammals have been found, albumin (Richardson et al., 1994) and transthyretin (TTR) (Kawakami et al., 2006; Santos and Power, 1999; Yamauchi et al., 1999). The latter protein appears to be the main TH carrier in fish and shows a great ability to bind T_3 (Morgado et al., 2006; Yamauchi et al., 1999) unlike mammalian TTR which preferentially binds T_4 (Chang et al., 1999). The biological significance and adaptive advantage of this transport system is largely unexplored in fish or even in higher vertebrates. As THBP is a major issue of the present work this matter will be further developed in the following section.

1.3.5 *Thyroid Hormones Binding Proteins (THBP)*

THs exert their action at a cellular level to bring about important physiological processes and therefore the delivery of THs from their site of synthesis to their site of action (target tissues) is a crucial process in vertebrates. Plasma is the immediate major compartment into which the thyroid secretes THs (mostly T_4) and the ultimate source of

THs for all target tissues. As previously mentioned, in vertebrate's less than 1% of THs are present in blood as the free form (physiologically active fraction) as the majority is bound to THBP. These proteins are synthesized by the liver and secreted into the bloodstream where they bind and transport THs. In the serum of larger mammals, the major THBPs are thyroxine-binding globulin (TBG), Albumin (ALB) and Transthyretin (prealbumin,TTR) (Larsson et al., 1985). In humans, serum albumin is present at approximately 100-fold the molar concentration of TTR and 2000 fold that of TBG. Although present in much lower concentration, TBG shows the highest affinity for THs (50 fold higher than TTR and 7000 fold higher than ALB) and carries about 75% of all plasma T_4 while TTR and ALB transport 20% and 5% respectively. Table 1 shows the main characteristics of these three THBPs. TBG is a 54KDa acidic glycoprotein (apparent molecular weight 60KDa in SDS-PAGE) which in addition to binding T_4 also binds T_3 but with slightly less affinity. It was first recognised as a major THBP in 1952 (Robbins, 1992) and its primary structure deduced in 1989 (Flink et al., 1986). The mature molecule without the 20 amino acids signal peptide is composed of 395 residues (44KDa) and four heterosaccharide groups. It has a single iodothyronine binding site and loses affinity for THs above 55ºC although association with T_4 increases its stability. Alteration of TBG synthesis or degradation processes leads to acquired TBG abnormalities (Refetoff et al., 1976). Human serum albumin (HSA) is a 66.5 KDa protein composed of 585 amino acids with no carbohydrates and forming three main domains. It is the main protein present in serum where it constitutes half of the total protein content. Albumin binds a wide variety of molecules (hormones, drugs, etc.) and thus the association with THs can be considered as non-specific. In spite of containing several iodothyronine binding sites only one has higher affinity for THs but even so it is considerably lower than TH binding to TBG and for the reason the contribution of

albumin to TH transport is relatively minor. TTR is an unglycosylated 55 KDa tetramer which is highly acidic. Despite being present in serum at much higher levels than TBG it plays a lesser role in TH transport and only 0.5 % of circulating TTR is occupied by T_4. Its normal concentration in serum (250 mg/l) corresponds to a maximal binding capacity of approximately 300 μg T_4/dl. TTR also binds a great variety of non-iodothyronine ligands like for example salicylates, penicillin or flavonoid compounds which shown particularly high affinity for the protein. TTR will be characterized in detail in section 1.4 where its features, functions and structural/functional evolution are described.

Although the biological advantage of transporting a high percentage of hormone in a bound, non-active form has not been extensively explored, the existing evidence suggests it has several important functions. THBPs are probably responsible for the maintenance of a large extrathyroidal thyroid hormone pool counteracting permeation of strongly lipophilic hormones into cells (Ekins et al., 1982; Mendel, 1989; Mendel and Weisiger, 1990; Mendel et al., 1987). The latter effect allows an even distribution of hormones to tissues and safeguards the body from the effects of abrupt fluctuations in hormone secretion. THBP can also serve as an additional protection against iodine wastage by imparting macromolecular properties to small iodothyronine molecules limiting their urinary loss (Chan et al., 1972). Furthermore, THBP may promote hormone cellular uptake by interaction with cell membranes and prevent the undue loss of hormones through metabolism and excretion (they prevent glomerular filtration due to the large size of the hormone-protein complex).

The relative abundance and hormone binding properties of THBPs are not conserved in vertebrates. In a series of studies, reviewed by Richardson (2002), serum samples from individuals representative of different vertebrate classes (mammals, birds, reptiles,

amphibians and also fish (Richardson et al., 2005)) were analysed for the presence of THBPs. Albumin was the only THBP present in all species studied, suggesting it may be the evolutionarily oldest TH carrier protein. In addition to albumin, TTR was also present in the blood of several homeotherms (birds and eutherian mammals), a condition controlled by THs. The simultaneous presence of the three THBPs seems to occur only in some eutherian species where TBG was also found in addition to TTR and albumin. The same studies suggest an apparent absence of TTR in the plasma of adult amphibians, reptiles and fishes, however its presence is well established for the early stages of development (Funkenstein et al., 1999; Kawakami et al., 2006; Richardson et al., 2005; Santos and Power, 1999; Yamauchi et al., 1993; Yamauchi et al., 1999; Yamauchi et al., 1998) which are characterised by surging TH blood levels (Hulbert, 2000). The previous findings strongly suggest a straight relationship may exist between the increase in THBP distribution capacity and TH requirements. Such capacity, which obviously increased during the evolution of vertebrates, could be related with the developmentally regulated expression of a gene coding for a new THBP with higher affinity than those already present in blood (Richardson et al., 2005). The presence of three THBP with different T_3 and T_4 binding capacities may result in a very robust buffering system enabling a highly effective homeostatic control of free hormone levels. This could also offer some explanation to observations that THBP are not essential for normal thyroid function and questions about their adaptive value. Clinical studies in humans show that wide fluctuations in THBP concentrations or its complete absence do not alter the hormonal economy or metabolic status of the subject (Lissitzky, 1990; Refetoff, 1989). Mutations that lead to the absence of albumin in humans (Kallee and Ott, 1992) and rats (Mendel et al., 1989) or TTR in mice (Palha et al., 1994) are associated with normal phenotypes and the organisms remain eutheroid (with normal

TH levels). These findings are highly suggestive that the THBP system may involve several functionally redundant components. However, the adaptive advantage acquired throughout evolution could rely on such redundancy. One deficient component of the system could be compensated by the others: under conditions of TH stress a buffering system with three carrier proteins would provide a strong selection advantage over a single protein system (Richardson, 2002; Schreiber, 2002a).

The efficiency of the hormone transport and delivery system is certainly crucial for thyroid hormone-dependent processes to take place. Nevertheless, many aspects of the activity of such physiologically important TH transporters and mechanisms by which they regulate TH bioavailability remain unclear and call for further investigation.

Table 1. Properties and metabolic parameters of the three main THBPs in human serum.
From http://www.thyroidmanager.org/Chapter3/3a-frame.htm.

		TBG	**TTR**	**ALB**
Molecular weight (KDa)		54*	55	66.5
Structure		Monomer	Tetramer	Monomer
Isoelectric point (pH)		4.2-4.6	5.35	4.7-5.2
Number of binding sites for T_4 and T_3		1	2	Several
Association constant Ka (M^{-1})	T_4	$1x10^{10}$	$2x10^{8}$	$1.5x10^{6}$
	T_3	$1x10^{9}$	$1x10^{6}$	$2x10^{5}$
Concentration in serum (mean normal,mg/l)		16	250	40 000
Relative distribution of T_4 and T_3 in serum (%)	T_4	75	20	5
	T_3	75	<5	20
In-Vivo Survival Half-life (days)		5	2	15
Degradation rate (mg/day)		16	650	17000

* Apparent molecular weight by SDS-PAGE is 60KDa

1.4 Transthyretin (TTR)

Transthyretin was identified for the first time in 1942 in human serum and cerebrospinal fluid (Kabat and Moore, 1942; Kabat et al., 1942; Seibert and Nelson, 1942) and was originally called prealbumin due to its greater electrophoretic mobility at pH 8.6 than albumin. In 1958, TTR was first recognized to bind thyroxine (Ingbar, 1958) and designated thyroxine-binding prealbumin (TBPA). Determination of the primary structure by Goodman et al in 1974 (Kanda et al., 1974), led to proposal of a new name transthyretin (TTR) which was adopted in 1981 (Robbins, 1976) and reflects its ability to transport thyroid hormones and retinol.

TTR research has developed over several decades and has been reviewed by different authors (Benson and Uemichi, 1996; Schreiber, 2001; Power et al., 2000a; Schreiber, 2002a; Schreiber and Richardson, 1997). In common with most plasma proteins TTR is synthesized and secreted by the liver (Dickson et al., 1982) and released into the blood stream. It is produced as a single polypeptide chain and folds into a globular protein being is present in plasma as a tetramer of four identical monomers (Blake et al., 1971; Gonzalez and Offord, 1971; Rask et al., 1971) that bind and transport THs. TTR's role as one of the THBP in the plasma is probably its best characterised function and the biological meaning of the transport network to which it belongs has been discussed in section 1.3.5. However, a number of other characteristics for this protein have also been observed and are now considered.

1.4.1 *RBP transport*

Another major function attributed to TTR is its ability to bind retinol-binding protein (RBP), the 21 KDa specific carrier of all-*trans* retinol (Blaner, 1989; Goodman, 1984) from liver storage sites to target cells and is important in vision and bone growth. The

presence of retinol bound to RBP is essential for the formation of a stable complex with TTR and crystallographic studies have established that two RBP molecules can bind one TTR tetramer (Monaco et al., 1995) and do not interfere with TH binding (van Jaarsveld et al., 1973). In mammals holo-RBP (retinol-RBP complex unbound to TTR) circulates almost entirely bound to TTR. It has been proposed that this binding serves to prevent filtration of the relatively small RBP molecule through kidney glomeruli (Kanai et al., 1968; Peterson, 1971). In TTR null mice the levels of plasma RBP and retinol were found to decrease (Episkopou et al., 1993). Surprisingly though, the mice remained healthy showing normal retinol levels in tissues, their embryos develop normally and relatively little RBP was detected in their urine (Wei et al., 1995). In light of these findings, the current understanding of retinol transport is not complete and the participation of TTR in it still requires clarification. Previous studies regarding the evolutionary onset of TTR synthesis suggested it may not be absolutely required for retinol transport (Richardson et al., 1994; Schreiber and Richardson, 1997). Moreover, RBP is known to be present in the blood of fish but it could only be isolated in the uncomplexed form (Berni et al., 1992; Shidoji and Muto, 1977). Although piscine holo-RBP shows binding ability to human TTR it displays an extremely low or inexistent affinity to piscine TTR (Folli et al., 2003). Structural studies show a high degree of conservation in TTR’s TH binding sites but in contrast a few amino acid differences may be responsible for an apparent lack of TTR-RBP binding in fish. This suggests conservation of TTR-TH binding in all vertebrates whereas TTR-RBP binding ability may have been acquired later in evolution.

1.4.2 *TTR as a stress and nutritional marker*

In mammals TTR is produced by the liver and is a typical negative acute phase protein as its levels strongly decrease in response to injury. The liver plays a crucial role in the adaptive alterations accompanying any stressful condition and is extremely sensitive to nutritional insults. In malnutrition TTR and also ALB and TBG are significantly reduced due to depressed liver synthesis (Ingenbleek, 1985). Moreover, TTR and RBP are the first plasma proteins to decline in parallel as a result of early nutritional deprivation (Ingenbleek, 1985; Ingenbleek et al., 1975). According to the free hormone theory ((Mendel, 1989; Robbins and Rall, 1960), see section 1.3.3) the suppression of TTR and RBP synthesis releases increased amounts of free ligands readily available to target cells. This negative acute phase feature of TTR is commonly used by clinicians to monitor the nutritional status of their patients. In fact TTR has been extensively characterised as a marker of malnutrition or stress (Brugler et al., 2002; Ingenbleek and Bernstein, 1999; Ingenbleek et al., 1972; Ingenbleek and Young, 1994; Ingenbleek and Young, 2002). Food restriction in fish has also been found to cause a dramatic reduction in TTR transcripts (Power et al., 2000b) as previously observed in rats (de Jong and Schreiber, 1987; Le Moullac et al., 1992). However, it is still not resolved if down-regulation of TTR in liver is directly caused by malnutrition or is also a consequence of reduced TH levels in plasma. The factors behind regulation of TTR production are still unclear but it seems likely that in common with other hormonal homeostatic processes the concentration of THs have a role, although studies are still required to clarify this matter.

1.4.3 *TTR synthesis in the choroid plexus*

In addition to being produced in the liver, high levels of TTR expression have also been reported in the choroid plexus (Dickson et al., 1985; Soprano et al., 1985), where cerebrospinal fluid (CSF) is produced. In fact, evidence shows that abundance of both chicken and rat TTR transcripts in the choroids plexus are far higher than that in the liver (Duan et al., 1991; Schreiber et al., 1990). TTR is the most abundant of all proteins (Dickson et al., 1986) synthesized by the choroids plexus and it is secreted exclusively towards the brain (Schreiber et al., 1990) and is the only THBP produced in the brain (Schreiber and Richardson, 1997; Schreiber et al., 2001) .

Most of the cerebrospinal fluid surrounding the brain is produced in the choroid plexus (Cserr, 1971) and its epithelial cells, where TTR is synthesized, constitute the blood-cerebrospinal fluid barrier. The functional significance of TTR in the brain is probably related to its TH binding capacity and it is proposed to be the brain THBP responsible for hormone distribution (Dickson et al., 1987a; Schreiber et al., 1990). A model for TTR and T_4 distribution within the blood-brain barrier was proposed by Southwell et al (1993). In contrast to the situation in the liver, TTR synthesis in the choroid plexus is constitutive and does not change during the acute phase response which also suggests different regulatory and functional requirements for TTR produced in the brain and in the rest of the body (Dickson et al., 1986).

1.4.4 *Molecular Structure*

Human TTR is a 55KDa homotetramer composed of four identical subunits, each containing 127 amino acids. The overall shape of the tetramer forms a narrow central channel which buries two identical thyroid hormone binding sites (Blake et al., 1974).

However, under physiological conditions only one of the binding sites is filled at one time due to negative cooperative effect of hormone binding (Neumann et al., 2001).

The three-dimensional structure of human TTR (fig. 5) was determined by X-ray crystallography (Blake, et al., 1978; 1974) at 2.5 Å resolution and has subsequently been refined by other authors (Hamilton et al., 1993; Hornberg et al., 2000; Wojtczak et al., 1992). In the first models the N-terminal region of the monomers could not be resolved but in 1993, in the structural model conceived by Hamilton et al. the first 10 N-terminal amino acids were observed as curve structures placed near the opening of the central thyroid hormone binding channel.

Presently, TTR X-ray structures from several mutant variants of human TTR (for review see Hornberg et al., 2000) and also from chicken, rat and fish are also available (Eneqvist et al., 2004; Folli et al., 2003; Sunde et al., 1996; Wojtczak, 1997). The models show little variation between structures pointing to an overall conservation of TTR three-dimensional structure during vertebrate evolution. X-ray diffraction indicates that the dominant secondary structure is the β-sheet. Each monomer (A, B, C and D) has two 4-stranded β-sheets and a short α-helix. Anti-parallel β-sheet interactions link monomers into dimers and a short loop from each monomer forms the main dimer-dimer interaction resulting in the formation of the central hydrophobic channel (fig. 5). The two TH binding sites situated between monomers A, C and B, D can be divided into three symmetry-related pairs of halogen binding pockets (HBP) comprised of an inner and outer cavity: HBP1 (HBP1'), HBP2 (HBP2'), HBP3 (HBP3'). The main difference between species is the lack of α-helix structures in the chicken TTR subunits. The TH binding site seems to be highly conserved with only one amino acid substitution in the sea bream sequence (Ser117 in the human sequence is substituted by a Thr). However, studies carried out by Eneqvist, et al (2004) point to differences in the

shape of the binding channel between sea bream and human TTR. In sea bream TTR the channel is wider at its entrance and has a narrower inner and outer cavity. Homology models based on the known X-ray crystal structure coordinates have been constructed for Lizard (Achen et al., 1993) and bullfrog (Yamauchi et al., 1998) TTRs and also for fish TTR (Power et al., 2000a) and subsequently X-ray crystallography was carried out. These models showed little variation from the known TTR structures. Despite the high conservation of the structures, comparisons of the electrostatic properties between the piscine homology model and X-ray structures from chicken, human and rat revealed that the surface potential, particularly in the TH binding site is noticeable more negative in chicken and even more-so in the sea bream structure than in human or rat (Power et al., 2000a).

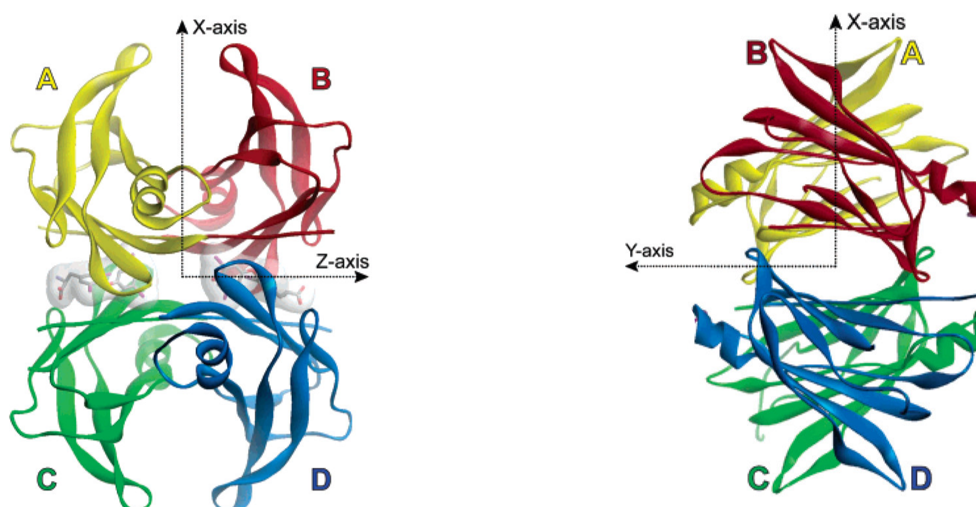

Figure. 5. Diagram of human tetrameric TTR crystal structure with each monomer colored differently (subunit A, yellow; subunit B, red; subunit C, green; subunit D, blue). TTR structure is predominantly β-sheet; each monomer has a two 4-stranded β-sheets and a short α-helix. A short loop from each monomer forms the main dimer-dimer interaction resulting in the formation of the tetramer. Left panel represents a view (perpendicular to the binding site, Z-axis) of the co-crystal structure of TTR in complex with T_4 (shown in stick representation) showing the two TH binding sites. Right panel presents a view (down the Z-axis) rotated by 90° about the Y-axis (relative to the view on left) to display the central channel where TH and other ligands bind. Original diagrams taken from Johnson et al. (2005).

1.4.5 *Genomic structure and transcriptional regulation*

The genomic structures of TTR have been described for human (Sakaki et al., 1989) rat (Fung et al., 1988) and mouse (Costa et al., 1986). Human TTR is encoded by a single copy gene located on human chromosome region 18q11.2-q12.1 (Sparkes et al., 1987). The gene spans 6.9 Kb with 4 exons, 3 introns and a TATA box like sequence and binding sites for HNF-1, 3 and 4 are located within 150bp from the transcription start site (Sasaki et al., 1985; Tsuzuki et al., 1985). The first exon codes for the 5' untranslated region, the 18 amino acid signal peptide and the first 3 residues of the mature protein. The second exon encodes residues 4-47 whilst exons 3 and 4 code for residues 47-92 and 93-127, respectively. A similar TTR gene organisation is found in both rat and mouse genomes.

TTR gene expression in the liver is under typical negative acute phase regulation. An extremely well conserved DNA-segment (CTGGGAA) in the 5'-flanking region (Fung et al., 1988) of the gene is thought to be important for such response during inflammation and trauma which causes TTR mRNA in liver to decrease dramatically. Further regulatory elements can be found at least 2000 bp upstream the gene that may be involved in regulation of expression via *cis* and *trans*-acting factors (Costa et al., 1989; Yan et al., 1990).

In contrast to the liver, in choroids plexus TTR gene expression does not respond to inflammation or trauma (Dickson et al., 1986; Dickson et al., 1987b). Thyroid hormone status also doesn't seem to influence TTR expression levels in the choroid plexus (Blay et al., 1993). Very little is known about the regulatory mechanisms of TTR gene expression in that region of the brain. In fish and amphibians where TTR expression seems to be mainly restricted to the liver, the genomic organisation has not yet been

determined. Its clarification would provide an important means for identification of 5' regions important for regulation of TTR expression in the brain.

1.4.6 *TTR Evolution*

Over the last two decades TTR synthesis and gene expression has been studied in many species. Also, the amino acid sequence of TTRs from different species has been determined directly or derived indirectly from the DNA sequence (see fig. 6). These findings have been important in defining changes in TTR which accompanied evolution of vertebrates. The changes identified encompass a number of different aspects such as, tissue expression patterns, TTR gene structure and TH binding properties.

1.4.6.1 *Evolution of TTR gene expression*

TTR has been identified in a large number of vertebrates, including eutherian mammals, marsupial mammals, birds, reptiles, amphibia and fish. As in humans, TTR gene expression in other eutherian mammals, marsupials and birds (for review see Power et al., 2000a; Schreiber, 2002a; Schreiber and Richardson, 1997) is mainly restricted to liver and choroids plexus. However, TTR synthesis was also found in the retina of cattle, rats and sheep, in lower levels in rat and human pancreas visceral yolk sack during fetal rat development and in developing chicken heart (for review see Power et al., 2000a; Schreiber et al., 2001). The ontogeny, tissue specific expression and function of TTR during development is poorly described. In reptiles TTR was found to be strongly expressed in the choroids plexus of lizards, turtles and young crocodiles but not at all in their livers (Achen et al., 1993; Schreiber, 2002a). In amphibians TTR mRNA was observed in the liver of premetamorphic tadpoles of frog (*Rana catesbiana*) and African clawed frog (*Xenopus laevis*) but no traces of TTR expression were detected in

the choroids plexus. Instead, lipocalin, a protein known to bind small hydrophobic ligands was found to be the main protein synthesized in that region of the brain (Achen et al., 1993; Yamauchi et al., 1998). In fish although small amounts of TTR mRNA were present in other tissues than liver (Funkenstein et al., 1999; Manzon et al., 2007; Power et al., 2000a; Santos and Power, 1999) synthesis in the choroid plexus was not found.

The evidence so far available appear to suggest that TTR expression in the choroid plexus first arose at the stage of stem reptiles but expression in the liver probably appeared in or prior to ancient fish. It has been hypothesised that the expression and synthesis of TTR in the choroid plexus of reptilians may be associated with the appearance of a neocortex (Kent, 1987). This idea has been further developed (Schreiber, 2002b) and the rapid increase in brain relative to body size and complexity during evolution has been proposed to exert a selection pressure for the appearance of TTR synthesis in the choroid plexus. As the blood-brain barrier restricts the access of THBP to the brain, a THBP produced within the brain would ensure an efficient distribution of THs essential for brain development and differentiation.

A number of hypotheses have been proposed for the onset of TTR synthesis in the liver (Richardson, 2002) and the consequent provision of an additional THBP in blood plasma. A first theory proposes that the selection pressure for hepatic TTR production was the increase in size of internal organs and consequently larger lipid pools. The appearance of a THBP with higher affinity for THs than albumin would help counteract their partitioning into tissues. Another hypothesis relates the evolution of TTR expression in the liver with homeothermy as THs are strongly involved with control of basal metabolic rate. This theory is supported by the production of TTR in the liver of birds, diprotodont marsupials and eutherians which are better homeotherms than

polyprotodont marsupials, monotremes, reptiles, amphibians and fish in which the capacity to synthesize hepatic TTR in adults has not been demonstrated (Richardson, 2002; Richardson et al., 2005). In both fish and amphibians TTR hepatic synthesis is proposed to be developmentally regulated and a consequence of the characteristic upsurge of TH levels in the blood during early development stages. However, recent studies in fish clearly demonstrate transcripts of TTR in adult fish liver (Power et al., 2000a), countering the "homeothermy" proposal.

1.4.6.2 *Evolution of TTR binding properties*

In addition to evolution of differential tissue expression of TTR a change in TTR binding affinity to T_3 and T_4 appears to have occurred during evolution. Such binding affinities have been measured for a number of different vertebrate species (Chang et al., 1999; Prapunpoj et al., 2002; Prapunpoj et al., 2000) revealing that in birds, reptiles and amphibians TTR seem to bind more strongly to T_3 than T_4 in contrast to the situation in mammals (see Table 2 for Kd values). These observations might suggest that during vertebrate evolution there was a change in TTR function from a T_3 transporter to a T_4 transporter. Also in fish few reports exist of TTR binding to both T_3 and T_4 but showing higher affinity for T_3 (Kawakami et al., 2006; Santos and Power, 1999; Yamauchi et al., 1999). The hormone binding affinity of piscine TTR is explored in chapter 3 of the present thesis and is a key goal of the present work (see chapter 3).

The implications of the changes in TH binding properties of TTR during vertebrate evolution are unclear but may be related to differences in TH balance and metabolism. For example, in fish there are several reports of higher blood levels of T_3 than T_4 (Bjornsson et al., 1998; Eales and Shostak, 1987; Pavlidis et al., 1997; Power et al.,

2000b) in contrast to the situation in mammals where T_4 is more abundant than T_3 (McNabb, 1992).

Table 2. Dissociation constants (Kd values) for TTR-TH binding from several species representatives of different vertebrates groups. Table source: Schreiber (2002b) and original values compiled from Chang et al. (1999), Prapunpoj et al. (2002; 2000). TTR from eutherians, marsupials and birds were purified from serum while crocodile and xenopus TTRs were produced by recombinant methods in the yeast *Pichi pastories*.

Source of TTR	Kd T_3 (nM)	Kd T_4 (nM)	Kd T_3/ Kd T_4
Eutherians			
Humans	56.6	13.6	4.2
Sheep	63.5	11.3	3.2
Rats	67.2	8.0	8.4
Marsupials			
Wombat	97.8	21.8	4.5
Possum	206.1	15.9	12.9
Wallaby	65.3	13.8	4.7
Birds			
Emu	18.9	37.4	0.51
Chicken	12.3	28.8	0.43
Pigeon	16.1	25.3	0.64
Reptiles			
Saltwater crocodile	7.56	36.7	0.21
Amphibians			
Xenopus laevis	248	508	0.49

This leads to the question of the evolution of T_4 as a pro-hormone in mammals and the associated evolution of deiodinases in TH target cells. The generation of T_3 from T_4 by deiodinase activity allows tissues specific regulation of TH action. Evolution of TTR expression and TH preferences may be correlated to the evolution of deiodinases and may represent an adaptation to the special needs in each species. Of particular interest is the brain-specific 5'-deiodinase (Type II deiodinase) in mammals which converts T_4 into T_3. A similar enzyme is present in other tissues but not in the brain of fish, amphibians or reptiles (for review see Schreiber, 2002b) and it has been suggested (Schreiber, 2002b) that the emergence of an efficient brain-specific deiodinase in

mammals could be related to the increase in TTR T_4 affinity. In fact, in mammals TTR only transports T_4 but not T_3 into the brain through the blood-cerebrospinal fluid barrier (Dickson et al., 1987a). The presence in birds and reptiles of TTR which preferentially binds T_3 suggests it may be the main hormone transported into the brain and may explain the absence of a type II deiodinase. The development in mammals of a brain-specific deiodinase system would provide an important adaptive advantage for control of cerebral TH homeostasis responding to the increase in relative brain size during evolution (Schreiber, 2002b; Schreiber et al., 2001).

1.4.6.3 *Evolution of TTR structure*

TTR genes have been cloned and sequenced in vertebrates from fish to mammals and in all cases only a single mRNA transcript has been found. The general cDNA structure consists of a 5'untranslated region (14-30 nucleotides), a coding region (127-130 amino acids) and a 3'untranslated region (115-181 nucleotides) preceding the poly(A) tail.

As the primary structures of TTR from different species became available attempts have been made to understand the relationship between structure, function and evolution. The determination of the three-dimensional structure of TTR tetramer allowed the establishment of correlations between the monomers polypeptide chain with their position on the tetrameric molecule leading to several conclusions. High primary structure homology is found at the interfaces of the subunits and the surface of the molecule seems to be less conserved than the interior region. The length of the mature monomer polypeptide chain varies from 127 amino acid residues in human TTR to 130 in teleost fish and 136 in agnatha fish (lamprey) (see fig. 6) and comparison between the sequences reveals 36 to 85% sequences identities (Manzon et al., 2007; Power et al., 2000a). TTR sequence in fish share the lowest identities and between teleost and

agnatha only 47% identity is found. However, there is an intriguingly high percentage amino acids identity throughout the polypeptide chain within classes and in fact

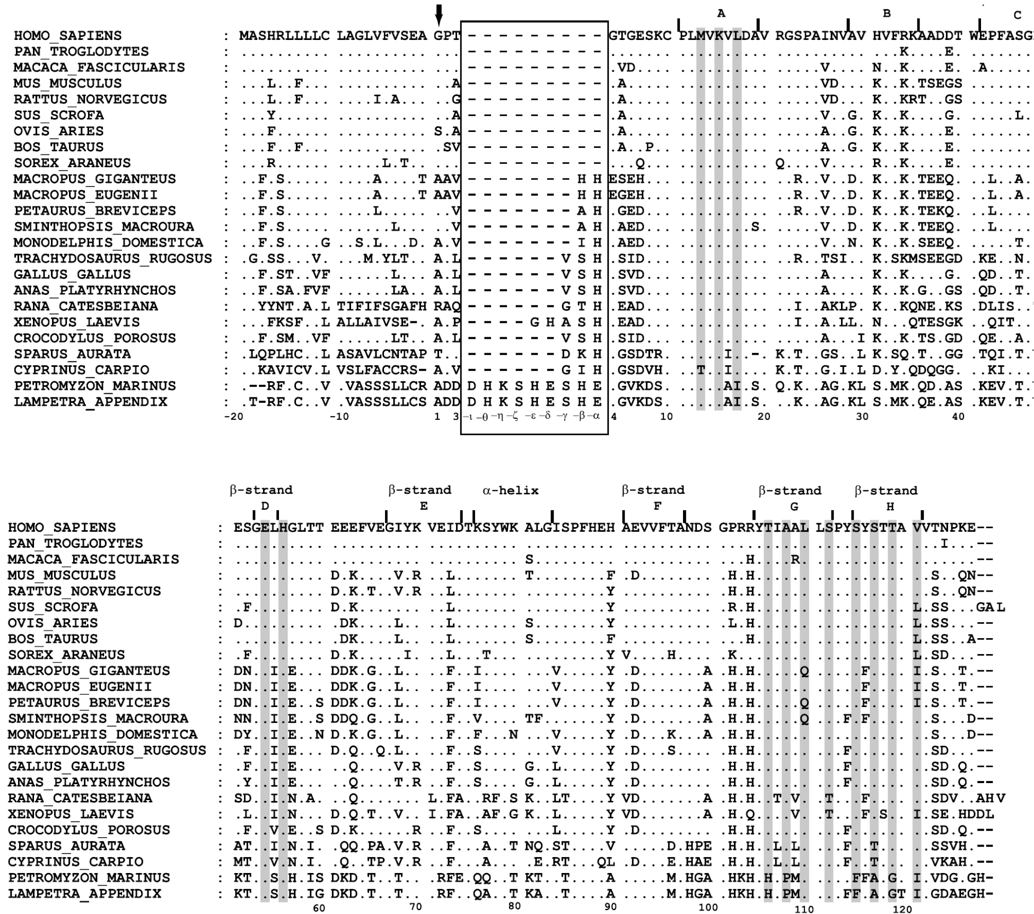

Figure 6. Multiple sequence alignment of TTR monomer amino acid sequence from representative species of all vertebrate groups with the following accession numbers: *Homo sapiens*, AAA73473; *Pan troglodytes*, Q5U7I5; *Macaca fascicularis*, Q8HXW1; *Mus musculus*, P07309; *Rattus norvegicus*, P02767; *Sus scrofa*, P50390; *Ovis aries*, P12303; *Bos taurus*, O46375; *Sorex araneus*, O46654; *Macropus giganteus*, Q29616; *Macropus eugenii*, P42204; *Petaurus breviceps*, P49142; *Sminthopsis macroura*, P49143; *Monodelphis domestica*, P49141; *Trachydosaurus rugosus*, P30623; *Gallus gallus*, P27731; *Anas platyrhynchos*, ABC65926; *Xenopus laevis*, BAA77579; *Rana catesbeiana*, P31779; *Crocodylus porosus*, O55245; *Sparus aurata*, AAC26108; *Cyprinus carpio*, CAD66520; *Petromyzon marinus*, DQ855960.; *Lampetra appendix*, DQ855961. The first amino acid of the mature TTR subunit is indicated with an arrow. Residues identical to the human TTR sequence in other species are represented by dots and features of secondary structure of the human TTR (Blake et al., 1978) subunit are given above the sequences. Amino acids located in the central channel and that are thought to be involved in hormone binding are shaded. The region where amino acids have been lost at the 5' end of exon 2 during the evolution of the TTR subunit is marked with a rectangle. Positive numbering of the amino acid residues denoted below the alignment refer to the mature human TTR sequence; negative numbers are

used to denote the signal peptide and greek letters represent the positions of residues absent in eutherians TTR sequence.(Manzon et al., 2007)

between all vertebrate sequences described so far. In particular the amino acids located in the TH binding channel region are almost 100% identical between different species. This high homology points to a strong selection pressure to maintain this structure counteracting variability due to mutations or genetic drift between populations and also seems to highlight the importance of TTR as a TH carrier during evolution. Nevertheless, evolution-related mutation can be found in the molecule and are quite unevenly distributed throughout the polypeptide chain. By far, the greatest frequency of amino acid changes is found at the N-terminal section of the subunits. This region stands out by its systematic change during evolution of both length and properties. It is shortest in eutherians, of intermediate length in the marsupials (with two additional residues) and longest in birds, reptiles, amphibians and fishes with three additional residues: Val-Ser-His in chicken and lizard; Gly-Thr-His in frog and Asp-Lys-His in sea bream (see fig. 6). Very recently the cDNA structure has been determined for TTR from the Lamprey which has an even longer N-terminus with 9 extra amino-acids Asp-His-Lys-Ser-His-Glu-Ser-His-Glu. The shortening of this section seems to lead to an increase in the hydrophilicity of its character. In the TTR gene, such loss of residues occurred at the border between exon1 and 2 and appears to results from a stepwise shift of the splice site at the 5’ end of exon 2 by a series of single base mutations converting codons into splice recognition sites (Aldred et al., 1997). It seems therefore that a sequence of small steps led to the shorter more hydrophilic N-terminal segment of the TTR subunit. Such an event likely fits in Darwin’s postulates of positive environmental adaptations (Aldred et al., 1997) in which slight functional improvements transmitted throughout generations give a selective advantage.

The functional adaptation arising from these N-terminal alterations is not completely clear, however, since changes in TTR binding strength to T_4 follow a similar unidirectional way in evolution these two features have been correlated. The hypothesis was that changes in the N-terminus were responsible for changes in binding affinity to THs (Schreiber and Richardson, 1997): a longer and more hydrophobic N-termini leads to preference for T_3 while a shorter more hydrophilic N-termini correlates to T_4 binding. The X-ray models predicted for TTR revealed that the four N-terminal regions are unstructured, protrude from the tetramer and are located near the entrance of the binding channel that harbours the TH binding sites. It could be possible that changes in the hydrophobicity of this regions influence their flexibility and therefore the access of T_4 to the binding site (Chang et al., 1999). To test these hypothesis previous studies involving the production of recombinant chimeric TTRs were carried out. A first study reported changes in TH affinity when an N-terminal section of crocodile (*C. porosus*) TTR was replaced by *Xenopus laevis* N-terminus (Prapunpoj et al., 2002). Recently binding to THs was tested using a human TTR chimera with the N-terminal region of crocodile (*C. porosus*) TTR and to a truncated crocodile (*C. porosus*) TTR lacking the N-terminus (Prapunpoj et al., 2006). It was proposed that binding to THs is in fact influenced by the character the N-terminus. Furthermore it was suggested that this region could influence T_4 binding preference whereas T_3 affinity would be mainly determined by the core of the molecule although further studies are required to clarify this matter. The precise contribution of the molecular electrostatic potential or the nature of the N-terminal section to TTR binding properties remains quite unclear and confused. Nevertheless, the evidence so far seems to point towards a coherent hypothesis of evolution of a TTR with a shorter hydrophilic N-terminal, preferentially binding T_4. This would provide an adaptive advantageous probably related to a more

efficient TH distribution system when integrated with the evolution of deiodinases (a matter previously developed in section 1.4.6.2).

In contrast to the uneven distribution of amino acid changes related to evolutionary adaptations another type of TTR gene mutation can be distinguished where amino acid substitutions randomly occur in TTR´s polypetide chain. About 100 of such mutations have been described in human TTR contributing to considerable sequence heterogeneity. Such mutation may be non-pathogenic but most of them are implicated in human pathologies associated with TTR amyloid fibril formation.

1.4.7 TTR amyloidosis

In common with other proteins with a high β-structure content, TTR is implicated in different forms of amyloidosis. Amyloid diseases are "late-onset, degenerative disorders characterised by the aberrant aggregation and extracellular deposition of proteins or peptide fragments" (Huff et al., 2003). Amyloid plaques contain fibrillar protein aggregates that results from misfolding and subsequent degradation of such proteins.

A number of human diseases are associated with this kind of processes like cystic fibrosis, Type II diabetes, Alzheimer's disease, Prion diseases (e.g. Creutzfeldt-Jakob), etc. (for review see (Dobson, 2003; Huff et al., 2003; Kirkitadze et al., 2002). Transthyretin amyloidosis are the most prevalent type of hereditary systemic amyloidosis. This form of the disease was firstly recognized by Andrade (1952) in a report of atypical peripheral neuropathy in Portuguese families. The syndrome was named familial amyloidotic polyneuropathy (FAP) and it is mainly characterised by the systemic deposition of TTR amyloidogenic variants in the peripheral nervous system causing a progressive sensory and motor dysfunction. The disease is invariably fatal

about 10 years after onset unless liver transplantation is performed during early stages (Ando et al., 1993; de Carvalho et al., 2002).

Since the first report of FAP many different kindreds with TTR amyloidosis have been identified (Benson and Uemichi, 1996). The most common clinical manifestations of TTR amyloidosis are familial amyloidotic polyneuroathy (FAP), senile systemic amyloidosis (SSA), familial amyloid cardiomyopathy (FAC) and the recently discovered central nervous system selective amyloidosis (CNSA) (see refs in Johnson et al. (2005)). SSA is an age-dependent sporadic disease resulting from misfolded of native TTR deposits in the heart (Westermark et al., 1990) whereas the familial diseases (FAP, FAC and CNSA) have been associated with tissue selective deposition (peripheral nervous system, visceral organs, autonomic nervous system, gastrointestinal tract, ocular tissues (Andrade, 1952)) of mutant TTR variants. In human TTR there has considerable sequence heterogeneity as a consequence of mutations resulting in single amino acids substitutions. There are over 100 identified TTR variants due to point mutations and more than 80 of them are pathogenic (for review see Benson and Uemichi, 1996; Connors et al., 2000; Eneqvist and Sauer-Eriksson, 2001; Sipe, 1992 and references in Johnson et al., 2005). One of the most clinically significant and frequent mutations is the substitution of methionine for valine at position 30 (ATTR V30M). While the site mutations do not seem to significantly change the native structure of the protein they appear to destabilize the tetramer in favour of fibril formation. The mechanism of TTR disassociation, misfolding and aggregation is not completely clarified but it is thought that tetramer disassociation is the rate-limiting step to amyloid fibril formation and that this process may be accelerated under acidic conditions (Colon and Kelly, 1992; Hurshman et al., 2004; Lai et al., 1996; Liu et al., 2000).

The current approach to prevent fibril formation mostly relies on stabilization of the native tetrameric conformation as a way to prevent the process of amyloidogenesis to commence (Hammarstrom et al., 2003; Klabunde et al., 2000; Miroy et al., 1996). *In vitro* studies have shown that binding of the natural ligand T_4 stabilized the tetramer reducing fibril formation (Miroy et al., 1996). During the past few years effort has been directed to developing TTR tetramer kinetic stabilization strategies (for review see Johnson et al., 2005a). Such studies relate tetramer stability to the TH binding site and involve identification of small molecules with high affinity for this region. This also highlights the importance of identifying the structural and physicochemical properties that influence TTR stability.

In spite of the high degree of identity between TTR sequences in vertebrates, TTR amyloidosis has not yet been described in non-human species. Even more intriguing is the fact that some of the pathogenic mutations in human TTR are normal features of the protein in other species. Sequence comparisons between pathogenic human TTR variants and other species reveal that 15 of the substituted amino acids have been 100% conserved in all other species with no pathogenic consequences (Power et al., 2000a). The high conservation of such residues suggests they might be of structural or functional importance. The fact that these mutations have no obvious effect in other species might indicate they arisen as a normal consequence of evolution. The reasons for these differences may be of great interest and remain unexplored. However the overall effect of any single amino acid mutation may depend on several structural, physicochemical and physiological factors that also differ between species. Thus, many variables would have to be taken into consideration making such analysis clearly complex.

1.4.8 TTR in fish

The first indications of the presence of TTR in fish came from the isolation of a protein in salmon (*Salmo salar*) (Larsson et al.) in 1985 on the binding of serum proteins to $[^{125}I]T_4$. However, subsequent attempts using serum binding assays to identify TTR in fish failed (Richardson et al., 1994) and its existence was only definitely confirmed in 1999 when TTR was isolated from masu salmon smolt serum (Yamauchi et al., 1999) and its cDNA (AAC26108) was cloned from sea bream (*Sparus aurata*) (Santos and Power, 1999). In 2003, the cDNA sequence for the common Carp (*Cyprinus carpio*) was also deposited in the sequence data bank (CAD66520) and in 2006 TTR was identified and its cDNA cloned from the Pacific Bluefin Tuna (*Thunnus orientalis*) (Kawakami et al., 2006). Very recently TTR was cloned and sequenced in two Lamprey, *Petromyzon marinus* (DQ855960) and *Lampetra appendix* (DQ855961) (Manzon et al., 2007).

The best characterized piscine transthyretin is sea bream TTR and the cDNA for sbTTR monomer contains an open reading frame of 630 bp encoding for a 130 amino acid preceded by a leader peptide of 20 residues (Santos and Power, 1999). The monomer and tetramer molecular weight are of approximately 14KDa and 55KDa respectively, as predicted for other vertebrates. sbTTR amino acid sequence shares 40-55% identity and 64-70% similarity with the sequences determined for other vertebrates (Power et al., 2000a). The X-ray structure of sbTTR has been determined (Eneqvist et al., 2004; Folli et al., 2003) revealing that there is very high overall structural homology and the TH binding region is conserved and a single amino acid substitution exists in this region (Ser117 in human to Thr in sea bream). Also, like in the human model, the N-terminal amino acids of sbTTR monomers formed disordered structures and could not be modelled. X-ray models of sbTTR in complex with T_3 and T_4 were also generated

(Eneqvist et al., 2004) and indicate that T_4 binds sbTTR in a similar mode to its binding to human TTR but the molecule doesn't reach so deep in the binding pocket. Also T_3 seems to bind the protein in a novel "reversed" binding mode. This study predicts that the sbTTR TH binding channel is similar but not identical to the human TTR showing a different shape and a significantly wider entrance. It is suggested that this small structural changes may explain the differences in T_3 and T_4 binding affinities proposed to exist between human and sbTTR.

Studies that characterise piscine TTR-TH binding affinities are very scarce. Yamauchi et al (1999) report after competition binding assays with $[^{125}I]T_3$ that masu salmon TTR binds T_3 with three times more affinity than T_4. A solid phase qualitative assay using non-denaturing PAGE and $[^{125}I]T_3$ $[^{125}I]T_4$ shows a strong signal for sbTTR T_3 binding (Santos and Power, 1999). sbTTR was also shown have high affinity for T_4 in fluorescence binding studies (Folli et al., 2003) but no quantitative values were provided. Recently, Kawakami et al. (2006) also report that the Pacific Blue Tuna TTR binds both T_3 and T_4, although no quantitative data was provided and the suggestion that piscine TTR has higher affinity for T_3 than T_4 has yet to be substantiated.

Another major function attributed to TTR is RBP binding. The RBP molecule has been cloned for several fish (Santos et al., 2002) and the amino acids known to be involved in the formation of RBP-TTR complex in higher vertebrates are partially conserved in fish (Folli et al., 2003; Santos et al., 2002). Also, binding affinity has been found between fish TTR and mammalian RBP (Berni et al., 1992; Folli et al., 2003). However, in fish no TTR-RBP complex has been found in the plasma so far (Berni et al., 1992; Zapponi et al., 1992) and binding studies found quite low affinity for both carp and sea bream TTR binding to RBP (Folli et al., 2003). The existence and importance of RBP transport by TTR in fish remains to be established.

Studies concerning TTR tissue distribution in fish suggest that in this group, in contrast to the situation in mammals, the presence of TTR transcripts is not restricted to the liver and choroids plexus. In juvenile sea bream, TTR is highly expressed in the liver but it seems to be absent in the choroids plexus (Santos and Power, 1999). Additionally TTR expression in juveniles was detected in smaller but significant amounts in other tissues like brain, intestine, kidney, testis and pituitary. In adult sea bream tissues, apart from the liver, significant TTR expression was also detected by Northern and Southern blot in intestine, heart, skin and muscle and in several other tissues (e.g. eye, gills, brain, pituitary, spleen, etc) in lower levels (Funkenstein et al., 1999; Power et al., 2000a). In bluefin adult tissues TTR transcripts were only found in liver and ovary (Kawakami et al., 2006) and in Lamprey TTR was again found in higher levels in the liver but also detected by RT-PCR in a wide variety of tissues as found in sea bream (Manzon et al., 2007). The physiological function of TTR in these tissues remains to be established but the coincident presence of TR in such tissues (except pituitary) suggests it may be related to TH transport system efficiency. Other functions may be involved but the widespread location of TTR expression clearly suggests evolutionary changes and adaptations.

In fish, very little is known about physiological conditions affecting TTR expression. The studies which exist are on sea bream and report downregulation of hepatic TTR expression by estradiol (Funkenstein et al., 2000) and in response to food restriction (Power et al., 2000b). In both cases decreased hepatic TTR transcripts seem to be coincident with lower circulating TH levels. High concentrations of estradiol in fish during the reproductive cycle have been associated with a reduction in TH levels (Norberg et al., 1989) and fasting is known to downregulate the hypothalamus-pituitary-thyroid axis in fish. In mammals TTR reduction in response to food restriction is well

established and TTR production in the liver is known to have a typical acute phase response regulation. It remains to be established if a similar process takes place in fish and the role of THs.

Studies about fish TTR are still very scarce and certainly merit more attention as the success, diversity and vastly differing life histories of fishes make them a very interesting group for studying the evolution of TTR function.

1.4.9 TTR binding to Endocrine Disruptors

Over the past years there has been increasing concern about the effect of synthetic chemicals on the endocrine system. A great number of chemicals are constantly released into the environment from industrial, medical or agricultural sources and are known to disrupt endocrine homeostasis in humans and animals (Colborn and Clement, 1992). Until recently most studies focussed on the reproductive system but evidence indicates that the thyroid axis is also vulnerable to endocrine-disrupting effects (Boas et al., 2006). Although little is known about their mode of action, disruption of thyroid homeostasis may take place at different levels, thyroid hormone synthesis, alteration in TH metabolism, cellular uptake, receptor binding and also by binding to THBP. In particular, interference with TH transport system may affect free hormone concentrations in plasma and therefore TH cellular uptake and physiological action (see section 1.3.5). In addition, binding of endocrine disruptor chemicals (EDCs) to THBP in plasma may also affect the free concentrations of such compounds modulating their access to cells.

In particular, TTR has been investigated as a target site for EDCs since it is able to bind exogenous compounds that structurally resemble THs. For example, binding to mammalian TTR has been shown for polychlorinated biphenyls (PCBs), hydroxylated

PCBs, dibenzo-*p*-dioxins, dibenzofurans or brominated flame retardants like polibrominated diphenyl ethers (PBDE) or tetrabromobisphenol A (TBBPA) (Brouwer, 1989a; Brouwer et al., 1989b; Brouwer et al., 1999; Brouwer et al., 1998; Brouwer and van den Berg, 1986; Cheek et al., 1999; Hamers et al., 2006; Lans et al., 1993; Meerts et al., 2000) in both *in vitro* and *in vivo* experiments. In such studies, TTR binding potencies to EDCs are comparable to, or higher than T_4. Furthermore, *in vivo* experiments suggest that the interaction of TTR with EDCs led to an increase in plasma clearance rates of T_4 resulting in decreased serum concentrations and causing hypothyroxinemia in rat, seal and humans. Also, evidence from animal models report disruption of TH homeostasis after exposure to PBDEs (for review see Darnerud et al., 2001) and such chemicals show an even closer structural relationship to T_4 than PCBs. The structural characteristics of EDCs greatly influence their affinity for TTR and it has been suggested that hydroxylation in *meta* and *para* positions would favour TTR binding (Lans et al., 1993). However, X-ray crystallography studies of TTR binding indicates that non-hydroxylated compounds can also bind strongly to the protein (Ghosh et al., 2000).

Until recently, most studies on the interaction of EDCs with THBP have been carried out with mammals. However, differences in the composition and binding properties of THBP among vertebrates (see sections 1.3.5 and 1.4.6.2) suggest different binding preferences for the chemicals and diverse impacts for thyroid system may occur. For instances, in larger eutherians the major THBP is TBG, which has no binding ability to EDCs (Lans et al., 1993; Rickenbacher et al., 1986; van den Berg, 1990). In rodents and lower vertebrates like birds, amphibians and fish, TTR is the major THBP (Richardson et al., 1994; Yamauchi et al., 1993; Yamauchi et al., 1999) and therefore binding to EDCs would likely have greater impact in this species. In recent studies, the effect of

EDCs binding to plasma THBP of chiken, bullfrog and masu salmon, pure plasma Japanese quail TTR and also recombinant TTRs from chicken, bullfrog was evaluated (Ishihara et al., 2003a; Ishihara et al., 2003b; Yamauchi et al., 2003). A great number of chemicals from different sources were potent competitors for T_3 (the natural TTR binder in such species) binding to TTR like for example, diethylstilbestrol, ioxynil, pentachlorophenol, bisphenol A, etc. Such *in vitro* competition assays may be useful tools for detection of EDCs in environmental samples in a species-specific manner (Yamauchi et al., 2003). This would be of particular interest for fish which are frequently used as sentinel species in aquatic environments which act as a sink for many pollutants.

In fish the binding ability of TTR to exogenous compounds is virtually unexplored, although numerous environmental contaminants have been reported to exert acute or chronic effects *in vivo* in teleosts (for review see Brown et al., 2004). The specific contribution of EDC interfering with TH transport for thyroid axis disruption in fish is difficult to determine as other thyroid endpoints would certainly be involved. Nevertheless, assessing the *in vivo* consequences of strong TTR binding *in vitro* would certainly be of great interest in fish, especially since thyroid disruption can severely compromise reproduction, development, growth and ultimately survival.

1.5. Aims and Outline

The general aim of the present thesis was to explore the structure/function characteristics of piscine transthyretin (TTR) and analyse its physiological role as a thyroid hormone binding protein (THBP). This was achieved by the following studies:

1. The molecular basis of TTR binding to thyroid hormones (THs) in fish and its structure/function evolution in vertebrates as a THBP.
2. TTR stability and fibril formation in fish.
3. TTR role and regulation, in particular in relation to THs in a fish model, the sea bream (*Sparus auratus*).
4. The affinity of fish TTR for xenobiotic compounds *in vitro* and its usefulness as a risk assessment tool in aquatic ecosystems
5. Fish thyroid axis disruption *in vivo* by binding of TTR to putative endocrine disruptors

In order to explore these topics it was first necessary to develop a number of different tools and methods. The experimental model used during this thesis was a teleost fish, the gilthead sea bream (*Sparus auratus*) which is from the Sparidae family. The sea bream has a high commercial value and has been extensively used as an experimental model to study growth, reproduction, physiology and endocrine regulation. Furthermore, characterisation of the thyroid system and potential endocrine disruption has mostly been conducted with teleosts. The cDNA sequence for sea bream TTR was available “in house” and the first tool developed was a sea bream recombinant TTR (sbrTTR) and several mutants in order to identify the role of the N-terminus in hormone binding. Other tools included the production of sbrTTR specific antisera, the establishment of a sbTTR specific ELISA and a sbrTTR binding assay. A none-

invasive, low stress and efficient method for administration of hormones and endocrine disruptors to fish was also established.

The results obtained in trying to achieve the proposed aims are herein described and were organized in different chapters as follows:

Chapter 3. Piscine Transthyretin hormone affinity and fibril formation: the role of the N-terminal. This chapter describes the production of sbrTTR and the characterisation of its binding affinity to T_3 and T_4. It also explores the putative role of sbrTTR N-terminal region on TTR binding affinity to THs and fibril formation through the production of two sbrTTR mutants were the N-terminus was truncated.

Chapter 4. Regulation of transthyretin by thyroid hormones in fish. In this chapter TTR plasma levels were measured for the first time in fish using a specific sbTTR developed ELISA. Furthermore, control of sbTTR expression and secretion by the THs was investigated upon *in vivo* treatment with T_3, T_4 and an antithyroid drug, methimazole.

Chapter 5. Disruption of thyroid hormone binding to sea bream recombinant transthyretin by ioxynil and polybrominated diphenyl ethers. In this chapter as specific sbrTTR *in vitro* competition binding assay was used to assess sbrTTR binding ability to a panel of putative endocrine disruptors.

Chapter 6. Disruption of the thyroid system by DES, Ioxynil in the sea bream (*sparus aurata*). In this chapter the *in vivo* consequences for thyroid system homeostasis by two chemicals found to bind TTR *in vitro* were investigated. Several endpoints of the thyroid cascade were addressed including TTR levels.

General Methods

This section gives a detailed description of the general laboratory techniques and materials used throughout the thesis. Many of the techniques described herein are common to several different chapters so to avoid repetition they are described in the present chapter. The methods section of each of the specific chapter gives an overview of the approach taken and also gives details of specific tasks.

Animal maintenance

Sea bream were obtained from TIMAR (Tavira, Portugal) and maintained at the experimental station Ramalhete Marine Experimental Station (University of Algarve, Faro, Portugal) in through-flow seawater tanks at natural annual conditions of water temperature, photoperiod and salinity. Maintenance and manipulation of fish were conducted in accordance with the recommendations of the Association of Animal Behaviour (ASAB, 2003) and the Guidelines of the European Union Council (86/609/EU).

Polymerase-chain reaction (PCR)

The polymerase chain reaction which uses a thermostable DNA polymerase from E.coli PVG-A1 is typically used to amplify DNA. In the present thesis it was used to amplify plasmid DNA and also cDNA prepared from mRNA extracted from tissue. A typical PCR reaction consisted of 1 µl of plasmid DNA dilution or cDNA (approximately 0.1ug), 15 pmol of each primer, forward and reverse, 20 µM each dNTP, 1.5-3 mM $MgCl_2$, 1U of *Taq* DNA polymerase (Promega, VWR, Portugal), 1x PCR buffer and molecular biology grade water (Sigma-Aldrich) to a final volume of 25 µl. Thermocycling conditions consisted of an initial denaturing step of 94 ºC for 3 min, followed by 15-40 cycles of 94 ºC for 45 s (denaturing), 59-69 ºC for 30-60 s (annealing) and 72 ºC for 50-180 s (usually 60 s/Kb expected, extension). A final elongation step was performed at 72 ºC for 5 min and PCR products were analysed by agarose gel electrophoresis (see appendix).

Cloning of PCR products

PCR products were purified directly from the PCR reaction mixture if a single product was detectable after electrophoresis. When multiple reaction products were detected upon electrophoresis the target product was excised from the agarose gel and purified using the GFX PCR DNA and Gel Band Purification kit (Amersham Biosciences). Purified DNA was eluted in 20-50 µl of sterile water (stH_2O) and 1 µl analysed on an agarose gel before cloning.

Cloning into pGEM-T Easy vector

The purified DNAs were cloned into pGEM-T Easy vector (Promega, VWR, Portugal) which is supplied linearized and has a single 3' thymidine (T) residue at both ends, which facilitates ligation to the 3'single deoxyadenosine (A) residues added to the PCR product by the 5´→3´ exonuclease activity of some DNA polymerases, including *Taq* polymerase (T/A cloning). Ligations were performed overnight at 4ºC using 15ng of vector, 10-200 ng of purified PCR product, 5 µl of 2x rapid ligation buffer, 1.5U of T_4 DNA ligase (Promega, VWR, Portugal, supplied with the vector) and stH_2O to a final volume of 10 µl. For transformation, 5 µl of the ligation reaction was mixed with a 100µl aliquot of *E. coli* XL1B competent cells, incubated on ice for 30 min and heat-shocked for 2 min at 42 ºC followed by 2 min on ice. Transformed cells were plated on LB agar plates containing ampicillin (75µg/ml) and isopropylthiogalactoside (IPTG, Sigma-Aldrich, Madrid, Spain 0.5mM) and 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-Gal, Merk Bioscience, Germany, 80 µg/ml) (see Appendix) for "blue/white selection" and grown overnight at 37ºC. The cloning vectors utilised contain the IPTG-inducible *lac* operon promoter followed by the multiple cloning site (MCS) located inside the coding region for the enzyme β-galactosidase. Recombinant bacteria are grown on plates with IPTG/X-Gal, and those containing a plasmid without an insert turn blue, as ligation results in an active β-galactosidase which degrades X-Gal and produces a

blue colour. Positive transformants with a DNA insert in the MCS of the vector will remain white, due to the interruption of the enzyme coding sequence which means that the active enzyme is not produced.

Cloning into pET 24b(+) expression vector

*pET e*xpression vectors (Novagen, Germany) are not supplied in a linearized form so preliminary steps of digestion and dephosphorylation are necessary. Digestion of the vector was performed, according to suppliers instructions (pET manual instructions), using 3 µg of pET vector, 3 µl of 10 x restriction enzyme buffer, 10-20 U of restriction enzymes (chosen to be compatible with insert restriction sites) and 3 µl of 1 mg/ml acetylated BSA in a final volume of 30 µl in stH_2O. Dephosphorylation was performed in a final reaction volume of 50 µl in stH_2O using 5 µl of 10 x alkaline phosphatase buffer and the right proportion of digested plasmid and enzyme: 0.05 U of shrimp Alkaline phosphatase per 1pmol of DNA ends (assuming that 3 µg of vector correspond to 4 pmol of DNA ends). The mixture was incubated at 37 ºC for 30 min followed by 15 min at 85 ºC for enzyme inactivation. Linearized vector was then separated from undigested circular vector by agarose gel electrophoresis and purified using the GFX PCR DNA and Gel Band Purification kit (Amersham Biosciences). Insert ligation was performed using 50-100 ng (0.015-0.03 pmol) of pET vector and approximately 30 ng (0.2 pmol) of insert, 1 µl of 10mM ATP, 2 µl of bovine serum albumin (BSA) 250 µg/ml, 2 µl of 10 x ligase buffer, 1 µl of T_4 DNA ligase (0.2-0.4 weiss U/µl) and stH_2O to a final volume of 20 µl. The reaction mix was then incubated at 16 ºC overnight.

Expression vector pET 24 was firstly transformed in XL1B *E. coli* cells. The purified DNA was sequenced using the dideoxy-terminator method (Seif et al., 1980) and an automated sequencer (Macrogen, Korea) in order to confirm the presence of the correct insert and the reading frame. Appropriate recombinant pET 24 expression vector constructs were used to

transform the host cells *E. coli* BL21DE3pLysS (Novagen). Transformation of *E. coli* BL21DE3pLysS with the expression vector was performed with recovery using the following protocol: 5 µl of ligation reaction was mixed with a 100 µl aliquot of *E. coli* competent cells (XL1B or BL21DE3pLysS), incubated on ice for 30 min and heat-shocked for 45 s at 42 ºC followed by 2 min on ice. Subsequently, 500 µl of LB broth (see appendix) was added and bacteria were grown for 30 min at 37 ºC with slow agitation (~1500 rpm). 100 µl of the culture was plated on LB agar plates containing the appropriate antibiotics for the plasmid (kanamycin) and the host strain (chloramphenicol) (see appendix) and grown overnight at 37 ºC. The pET vectors do not contain a *lac* operon promoter for "blue/white colour selection" so X-Gal and IPTG were not added to the plates.

Small-scale plasmid purification

In order to characterize positive transformants, plasmid DNA was isolated using the alkaline-lysis method (Sambrook *et al*. (1989). Single colonies were inoculated into 2 ml of LB broth containing 80 µg/ml ampicillin (see appendix) and grown overnight at 37ºC with vigorous shaking. 1.5 ml of culture was spun for 1 min in a microcentrifuge at maximum speed and the pellet resuspended in 100 µl of ice-cold GTE buffer (see appendix). To induce cell lysis, 200 µl of a freshly prepared solution of 0.2 N NaOH / 1 % SDS were added, mixed by inversion and incubated 5 min on ice. 150 µl of ice-cold 3M KAc (see appendix) were added to neutralize the lysate, mixed by inversion and incubated on ice for 5 min. Cellular debris were spun down for 10 min at maximum speed and the supernatant transferred to a fresh tube. RNase A was added to a final concentration of 20 µg/ml and incubated for 20 min at 37 ºC. An equal volume of phenol pH 8.0: chloroform (v/v 1:1) was added, mixed by vortexing and then centrifuged for 2 min at 7500 g at room temperature. The supernatant was precipitated with ethanol and resuspended in 50 µl of stH_2O. When a DNA preparation of higher quality

was required (e.g. for sequencing reactions) the Wizard Plus Miniprep DNA Purification System (Promega, VWR, Portugal) was used according to the manufacturer's instructions.

Restriction digest

The success of a range of different DNA manipulations carried out in the context of the thesis was assessed using restriction digest. This permitted the appropriateness of plasmid constructs and sequence insertions to be assessed based upon the restriction pattern obtained. A typical restriction digest included 1μl of purified plasmid, 0.5 μl of each enzyme (approximately 5U), 2 μl of 10 x compatible enzyme buffer (Promega, VWR, Portugal) and stH_20 to a final volume of 20 μl. The reaction products from restriction digest were analysed on 1% agarose gels (appendix).

Production of pure recombinant sbTTR

This procedure was thoroughly developed and optimized as it has never previously been carried out with fish TTR and was the basis of several techniques developed and utilised in the context of the thesis. The methodology was initially developed and applied to the wild type protein and was then scaled up and further used to produce mutant TTRs which lacked a differing number of amino acids. The process of recombinant protein production comprised a number of different steps:

Protein expression in E.coli

Recombinant protein expression was carried out using competent *E. coli* strain BL21DE3pLysS (Chloramphenicol resistant) transformed with the appropriate expression vector construct: pET24d(+)/sbTTRWT, pET24b(+)/sbTTRM6 or pET24b(+)/sbTTRM12. Transformation was performed using the heat-shock method and bacteria were plated on LB

agar plates containing 30 μg ml^{-1} kanamycin and 34 μg ml^{-1} chloramphenicol. For protein expression a pre-culture was prepared by placing a single colony in 10 ml LB broth with the appropriate antibiotic and growing overnight at 37 ºC. The pre-culture was then diluted in LB broth to a final optical density (OD_{600nm}) of 0.05 in a large scale culture (500 mL of LB broth) and grown at 37 ºC with vigorous agitation (250 rpm) until the OD_{600nm} reached 0.6-1.0 (approximately 2 h). At this stage protein expression was induced by adding IPTG (0.2 mM) to the culture and incubating it for a further 3 h under the same conditions. Bacteria were harvested by centrifugation at 4500 g for 20 min and the pellet frozen at -80 ºC before inducing cell lysis. Frozen bacteria were thawed and resuspended in 10 mL double distilled water containing phenylmethylsulphonylfluoride (PMSF) 1mM (Sigma-Aldrich). Lysis was induced by adding approximately 1 mg lysozyme (Sigma-Aldrich) plus 1mM magnesium chloride ($MgCl_2$) and incubating on ice for a further 10 min. DNase I (5U/μL, Amersham Biosciences) was subsequently added followed by incubation for another 10 min. Supernatant and cell membranes were separated by centrifugation at 25 000 g for 15 min at 4 ºC. The supernatant, containing the desired protein, was collected and stored at –80º C until required.

Protein purification by preparative electrophoresis (Prep cell 491 system)

Purification of the wild type and mutant proteins was achieved using a preparative continuous-elution electrophoresis system (Model 491Prep Cell, Bio-Rad laboratories). Optimization of this process was primarily carried out under reducing conditions (SDS-PAGE) in which a first pure sample of sbTTRWT was obtained and used for antibody production. However, when the goal was binding affinity studies, proteins were always purified under native scaled-up conditions to generate a higher concentration of proteins which bound the thyroid hormone (THs). Initial experiments revealed that production of TTR under denaturing conditions generated abundant protein but they lacked the capacity to bind

THs. In both cases preparative electrophoresis was carried our following the supplier's instruction manual for correct gel casting, assembly and operation (see appendix).

The general procedure for electrophoresis was as follows: the supernatant containing the recombinant protein was loaded onto a cylindrical preparative native Ornstein-Davis polyacrylamide gel (Davis, 1964; Ornstein, 1964) after mixing with the proper sample buffer (appendix). Electrophoresis was carried out at a constant power of 12 W over several hours (depending on gel size and running conditions) in running buffer (appendix) maintaining a constant temperature (18 ºC). Proteins fractionated on the polyacrylamide gel were allowed to migrate off the end of the gel, passed into an elution chamber and collected as individual 2.5 ml fractions in elution buffer (same as running buffer) at 8 ^{o}C (Valkonen et al., 2001) using a fraction collector (LKB 1, Pharmacia Biosciences). Initially the approximate volume at which TTR should elute was determined using analytical SDS-PAGE (15% polyacrylamide). In subsequent purifications fractions were collected which were expected to contain TTR and alternate fractions were analyzed by analytical SDS-PAGE (15% polyacrylamide) and silver stained in order to identify those containing the purified sbTTR. Fractions containing pure sea bream TTR were pooled, concentrated by centrifugation and dialysed against 50 mM Tris, pH 7.5. If the final sample was found to still contain contaminants it was subjected to a second round of purification using the 491 Prep cell.

Protein concentration

Protein fractions were concentrated using filtration columns Ultrafree 15, Biomax 5 MWCO (Millipore, UK) by centrifugation at 1900 g at 4 ºC. Before each centrifugation step the columns were filled/refilled with TTR containing fractions and in the final step the desired value was recovered. Finally, protein samples before and after concentration were analyzed by SDS-PAGE to confirm that this step was effective. Samples of filtrate were also included

in the final analysis as a control to ensure that significant amounts of protein were not lost in the filtration/concentration process.

Protein dialysis

The final purified protein sample was then dialysed at 4 °C in different fractions against Tris buffer 50mM pH 7.5 prepared with double distilled water. The use of native conditions and this dialysis step was essential to ensure that the protein retained its native tetramer conformation and therefore its ability to bind the hormone. Dialyses membranes (Sigma-Aldrich) with a molecular cut off (12 KDa) slightly under TTR's monomeric molecular weight (14 KDa) were chosen to ensure retention of the protein and to permit removal of contaminating small molecules like glycine (present in 491 Prep cell elution buffer) that might interfere with the protein binding ability in binding assays. Prior to dialysis membranes were prepared by first boiling for 30 min in a 100 mM NaHCO3 + 1 mM EDTA solution, followed by thoroughly washing with distilled water. The prepared dialysis membranes were then stored at 4 ºC in EDTA (1mM) for several months and utilised as required. Dialysis tubing containing the sample were immersed in over 1000 x excess buffer with slow agitation at 4 ºC during approximately 24 h. The dialysis buffer (50 mM Tris) was changed at least three times every three hours and then left overnight. Samples were recovered after dialysis and analysed by SDS-PAGE to ensure TTR was not lost during this process.

Protein quantification

The concentration of the purified protein was determined using a modification of the Lowry method (Lowry et al., 1951) and the molar extinction coefficient of the recombinant protein at 280 nm ([ε (280nm) (M^{-1} cm^{-1}) = (number of Trp) (5500) + (number of Tyr) (1490) + (number of Cys) (125)] (Pace et al., 1995).

Gel electrophoresis

SDS-PAGE

Protein analytical electrophoresis was carried out as described in the literature (Sambrook, 1989). Based upon the molecular weight of monomeric TTR optimisation of electrophoresis was carried out by varying the concentration of polyacrylamide between 12-15%; it was found that a resolving gel of 15 % polyacrylamide and a stacking gel of 5 % were the most appropriate for good resolution (see appendix for gel preparation reagents and volumes). Minigels of 1,5 mm thickness were prepared in a dual gel vertical caster system (Mighty Small^{TM} 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 (see appendix) at constant power of 40 mA. Prior to electrophoresis samples were prepared in 2x loading buffer containing DTT (see appendix) and boiled for 5 min at 100ºC to ensure protein denaturing. A prestained protein ladder (#SM0671, Fermentas) was always included in electrophoresis to give an estimate of the molecular weight of the proteins analysed. When the sample front reached the bottom of the gel during electrophoresis, the power was stopped and the apparatus disassembled and gels removed and stained. The stacking gel was removed using a scalpel blade and the resolving gel was carefully transferred to a glass or plastic box for staining.

Native PAGE

The proteins were also analysed by native PAGE to compare electrophoretic patterns with human plasma albumin. For native PAGE, polyacrylamide gels were assembled in a similar fashion but this time a 12.5% native glycine-acetate resolving gel (see appendix) was prepared and no stacking gel was added. Electrophoresis was carried out with a constant

current of 60 mA in glycine-acetate 1 x running buffer (see appendix). Detection in native gels was always done by silver staining.

Coomassie brilliant blue gel staining

Coomassie brilliant blue R is a sensitive protein stain which is used to locate protein bands after electrophoresis in polyacrylamide gel. The use of Coomassie blue staining was based upon the expected concentration of protein in the samples analysed. When a concentration greater than 0.5 µg/band was expected gels were immersed in a solution of Coomassie brilliant blue (0.25%) in a staining vessel for at least one hour at room temperature with slow agitation. The gels were then rinsed to remove excess coomassie blue and destaining solution (see appendix) was added and gels gently agitated for 4 hours to overnight with several changes of solution to remove excess coomassie dye from the gel.

Silver nitrate staining

Silver staining is a much more sensitive method than Coomassie brilliant blue and relies on the production of silver atoms from silver salts by groups present on the protein. The main problem with this method is that it is prone to artefacts and all the procedure must be carried out with clean and dedicated equipment. For this procedure clean glass material was always used and reagents prepared with deionised water to avoid silver nitrate precipitation. Gels were previously placed for several hours or overnight in a 50% methanol solution in a glass tank and then rehydrated in deionised water. A staining solution (see appendix) was prepared with silver nitrate and the washed polyacrylamide gel was incubated with vigorous agitation for 15 min. The gel was washed 3 to 4 times in deionised water and then transferred to developing solution (see appendix) and incubated with agitation until protein bands with the desired intensity were detected. The reaction was stopped in a 1% acetic acid solution.

Gel Drying and storage

Upon appropriate staining and analysis, polyacrylamide gels were carefully transferred to absorbent filter paper (Whatman paper), covered with cling film and dried using a gel dryer apparatus (Drygel.jr se540, Hoeffer Scientific Instruments, UK) under vacuum for one hour at 60-80 ºC. When a gel image was necessary, prior to drying gels were scanned and a digital file generated for storage.

Antibody Production

The production of polyclonal sbTTR antibodies was carried out by a commercial company, Agrisera, (Sweden). Purified recombinant sbTTR (~ 1mg in water) was sent on ice to Agrisera and was used to immunize a female rabbit using the standard protocol (www.agrisera.com). In brief, the rabbit was immunized 4 times with recombinant protein (~250μg per immunization) in 10mM Tris solution pH 7.5, over 14 weeks. After the third immunization a small blood sample was collected and serum was tested together with a sample of pre-immune serum (as a negative control) by Western blot to assess the titer and specificity of the antisera for seabream TTR. As the serum sample contained a high titre of antibodies immunoreactive with sbTTR the rabbit was bled and a larger volume of serum was collected.

Western blot: colorimetric and chemiluminescence analysis

Protein samples were fractionated in duplicate by SDS-PAGE as described above. One of the gels was stained with Commassie blue and the other 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, the stacking gel was removed and the resolving gel was marked by making a small cut in one corner and the dimension of gel measured with a ruler. A sheet of Hybond-P membrane was cut to the size of the 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 was prepared it was also soaked in protein transfer buffer (appendix) for 10-20 min. After equilibration a transfer "sandwich" was assembled by placing the Hybond-P membrane in close contact with the gel (ensuring no bubbles were present) and then placing 6 sheets of absorbent Whatman paper on each side. The "sandwich" (soaked in transfer buffer) was fastened into an electroblotting cassette and placed inside a vertical electroblotting tank (TE22 Mighty small tank transfer, Hoeffer, San Francisco, USA) filled with transfer buffer, ensuring that the gel was facing the negative electrode. After transfer, carried out for 1 h at 300mA with cooling, the Hybond-P membrane containing the transferred proteins was removed from the cassette, rinsed in PBS (appendix), wet in 100% methanol and washed for 5 min in distilled water before incubation in skimmed milk blocking solution (see appendix) overnight at 4 ºC with constant agitation.

Immunoblotting and detection with a chromogen

For immunoblotting the blocked membrane was then washed in 1x PBS for 10 min to remove excess blocking solution and incubated with rabbit anti-seabream TTR sera (optimized dilution of 1:10 000) for 2h at room temperature. Excess antisera was removed by washing membrane 3 x 5 min in PBS/Tween 20 washing solution (appendix) prior to addition of the secondary antibody (anti-rabbit IgG-peroxidase anti-peroxidase 1:5000). Membranes were incubated with the secondary antisera at room temperature for 1 h and washed 2 x 10 min in

washing solution prior to development with a freshly prepared 3,3'-diaminobenzidine (DAB) solution (0.04 %w/v) containing 0.003 % H_2O_2 and also 0.02 %(w/v) nickel chloride to intensify the signal. This step was carried out in the dark with agitation until appropriate signal intensity was attained and the color reaction was stopped by transferring the membrane to water.

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 (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 (see appendix) 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 respectively to allow a final volume of 0.1 ml/cm^2 of membrane. The membrane was placed in a sheet of clingfilm (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 (Kodac fixer and developer, 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.

Ligand binding assay

Development of sbrTTR $[^{125}I]$-T_3 binding assay was preceded by a long period of optimization in which trials were carried out in order to establish the assay buffer (concentration and pH), the sbrTTR protein concentration, incubation time and temperature, tubes, assays volume and separation method. Several methods were tested to achieve an efficient separation of free and bound phase: by gel filtration as previously described for human TTR (Almeida and Saraiva, 1996; Hamers et al., 2006; Lans et al., 1993) using 1ml Biogel P-6PG columns, using charcoal as adsorbent and finally the PEG method utilised with amphibian TTR (Yamauchi et al., 1993). The latter method was found to be most suitable for the sbTTR binding assay but had to be optimized. The optimal sbTTR protein concentration used in the assay was determined by titration. Several concentrations of sbrTTRWT (0.05, 0.15, 0.25 and 0.5 µg) were incubated with 0.1 nM $[^{125}I]$-T_3 in the presence or absence of an excess of 10 µM unlabeled T_3 (to assess non-specific binding) to determine binding percentages. A concentration per tube of sbTTR of 50 ng was found to be sufficient to permit a working binding percentage of at least 10 %.

As for incubation times and temperature; a range of temperatures, on ice, 4 ºC, room temperature (24 ºC) and 37 ºC were tested using 30 min, 1, 2, 4 hours and overnight

incubation. It was observed that 2 h on ice gave maximal binding and that longer incubation did not increase the amount of hormone bound. It was also found that the use of glass tubes was essential to allow proper binding conditions and with polystyrene or polypropylene plastic tubes the assay was not feasible. The use of double distilled rather than single distilled or deionised water was also a required condition as has previously been shown (Chang et al., 1999).

The overall best working conditions were selected to achieve the final assay protocol which is subsequently described and which was most like the assay condition proposed by (Yamauchi et al., 1993) for bullfrog TTR and also applied to other species (Ishihara et al., 2003) such as the fish, masu salmon (Yamauchi et al., 1999).

For competitive binding studies, sbrTTR (50ng per tube) was incubated in 200µL TCN buffer (see appendix) containing tracer 0.1nM $[^{125}I]$-T_3 in the presence of increasing concentrations of unlabeled ligand (0-10 µM) for 2 h on ice. Separation of free and sbrTTR bound $[^{125}I]$-T_3 was carried out on ice by adding to assay tubes 25 µL of 125 µg/ml bovine γ-globulin and 250 µL of 25 % (w/v) and PEG 6000 containing 0.2 M $ZnCl_2$ in TCN buffer (Yamauchi et al., 1993). The mixture was immediately centrifuged at 1500 g for 10 min at 4 ºC and the resulting pellet washed with 1ml of 12.5 % (w/v) PEG 6000 containing 0.1 M $ZnCl_2$ and counted in a gamma counter (Wizard, Pharmacia-LKB). Non-specific $[^{125}I]$-T_3 binding to rsbTTR was determined by incubating with an excess of unlabeled T_3 (10µM). Binding assays were carried out in duplicate and repeated on at least three different occasions. The data obtained was analysed using One-way analysis of variance (ANOVA). If statistically significant differences (considered at $P<0.05$) were detected between treatments, a Tukey (HSD) multiple comparison test was applied. All the statistical analysis was performed using Sigma Stat software version 3 (SPSS).

Thyroid hormone radioimmunoassay

Thyroid hormone (T_3 and T_4) plasma concentrations were measured using a specific and reproducible radioimmunoassay (RIA). Briefly, plasma samples were diluted (1/10) in phosphate buffer (0.01M pH 7.60), heat extracted (1h 30 min at 70ºC) then 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 T_4 sera (1:8000, ~3% cross-reactivity with T3; T2652 - Sigma-Aldrich, Madrid, Spain) or anti T_3 sera (1:15000, <0.01% cross-reactivity with T4; T2777 - Sigma-Aldrich) and 100 µl of tracer ($[^{125}I]$-T_4 and $[^{125}I]$-T_3, 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 10 min, 1500g at 4ºC. The supernatants were decanted, and the pellets were counted in a gamma counter (Wizard, Pharmacia-LKB).

Piscine transthyretin hormone affinity and fibril formation: the role of the N-terminal

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Abstract

Thyroid hormone binding proteins (THBP) are poorly characterized in fish and in the present study thyroid hormone (TH) binding by piscine Transthyretin (TTR) was determined, as was its ability to form fibrils. Piscine TTR, in common with mammalian TTR is a homotetrameric protein with two identical, highly conserved TH binding sites. However, piscine TTR has a longer N-terminus and the latter has been proposed to influence TH binding affinity between species and may also influence tetramer stability. In order to investigate this matter, wild type sea bream recombinant TTR (sbrTTRWT) plus two recombinant N-terminal mutants (sbrTTRM6 and M12, in which the first 6 and 12 amino acids respectively were removed) were produced and purified. Binding of $[I^{125}]$-T_3 to sbrTTRWT and mutants was confirmed by native polyacrylamide gel electrophoresis followed by autoradiography. Ligand binding studies revealed that both T_3 and T_4 bind sbrTTRWT with similar affinities (Kds of 10.6 ± 1.7 nM and 9.8 ± 0.97 nM, respectively) and that the mutation sbrTTRM12 had no significant effect on T_3 and T_4 binding affinity. However, on removal of the 6 N-terminal amino acids (sbrTTRM6), poor affinity for T_4 was found (Kd = 252.3 ± 15.8 nM) suggesting that the N-terminus does influence the binding characteristics of sbTTR. The latter mutation was also found to inhibit amyloid fibril formation *in vitro* under acidic conditions as shown by fluorometric measurements using the fluorescent dye thioflavine. Such studies also suggested that sbrTTRWT is more resistant to acid-mediated fibril formation than the human TTR.

Introduction

Transthyretin (TTR) is one of several thyroid hormone binding proteins (THBP) which transport thyroid hormones thyroxine (T_4) and triiodothyronine (T_3) in the circulation of vertebrates. This protein is synthesized and secreted mainly by the liver and its functionally active form is a tetramer, the central core of which is proposed to transport one or two molecules of thyroid hormones (THs) (Blake et al., 1978; Schreiber and Richardson, 1997; Wojtczak et al., 1996). Subsequently TTR was also identified in the choroid plexus of mammals where it is proposed to play an important role in the transport of T_4 across the blood brain barrier (Blay et al., 1993; Hamberger et al., 1990; Harms et al., 1991; Schreiber et al., 1990).

TTR has now been identified in most vertebrate groups; it is present in placental mammals, Australian and American marsupials, birds, reptiles, amphibians and fish (Dickson et al., 1985; Duan et al., 1991; Duan et al., 1995; Larsson et al., 1985; Mita et al., 1984; Prapunpoj et al., 2002; Prapunpoj et al., 2000; Richardson et al., 1994; Richardson et al., 1997; Santos and Power, 1996; Santos and Power, 1999; Yamauchi et al., 1993; Yamauchi et al., 1999). The distribution of TTR in blood, liver and the choroid plexus of representative species of these groups are variable, making it difficult to establish a consensual model for the evolution of this gene. For example, TTR is present principally in the liver and to a lesser extent in the brain and other tissue of teleost fish (Power et al., 2000a; Santos and Power, 1996; Santos and Power, 1999) but in turtles (*Trachemys scripta*) it is expressed principally in the choroid plexus (Richardson et al., 1997). Moreover, some metatherian (marsupial) species contain TTR in the blood stream, whereas others do not (Richardson et al., 1994). In addition to differences in tissue distribution of TTR between species, differences in the thyroid hormone binding properties of the protein have also been observed.

The TTR tetramer is a highly stable protein, however dissociation and misfolding of this protein can lead to its conversion into insoluble amyloid structures that can deposit extracellularly. This process is associated with severe amyloid disease in humans: familial amyloid polyneuropathy (FAP) and senile systemic amyloidosis (SSA) (reviewed in (Benson and Uemichi, 1996; Ingenbleek and Young, 1994; Schreiber and Richardson, 1997; Sipe, 1992). Tetramer dissociation seems to be the critical step in misfolding and *in vitro* studies have shown that the TTR tetramer can form fibrils under acidic conditions at 37 ºC (Hammarstrom et al., 2001; Lai et al., 1996).

The resolution of the crystal structure of human, chicken and rat TTR has revealed that the functional tetramer contains two structurally identical binding sites in the central channel (Blake et al., 1974; Hamilton et al., 1993; Sunde et al., 1996; Wojtczak, 1997; Wojtczak et al., 1992). The amino acids that are thought to be involved in T_4 binding in the central channel of TTR appear to have been conserved between mammals and lower vertebrates. However, binding studies of plasma TTR with radiolabelled T_4 and T_3 have established that mammalian TTR has the highest affinity for T_4 while avian TTR preferentially binds T_3 (Chang et al., 1999). Similar preferences for T_3 were found for crocodile (Prapunpoj et al., 2002) and amphibians (Prapunpoj et al., 2000; Yamauchi et al., 1993). The molecular basis for the different affinity of mammalian and lower vertebrate TTR for the thyroid hormones remains to be established but it has been proposed that changes in the N-terminus of the protein may be an important factor. This region is the least conserved between species and in birds, reptiles or amphibians it is longer, having three additional amino acids, and is more hydrophobic than mammalian TTR (Aldred et al., 1997).

The identification of TTR in teleost fish is relatively recent, the protein was isolated and the N-terminus sequenced from juvenile Masu salmon (*Onchorhynchus masou*) plasma (Yamauchi et al., 1999) and a full-length cDNA for seabream TTR was isolated from the liver

of juvenile seabream (Santos and Power, 1996; Santos and Power, 1999). Binding studies with Masu salmon TTR revealed that it preferentially binds T_3 (Yamauchi et al., 1999). The crystal structure of sea bream TTR has been determined and showed that the overall topology of seabream TTR is conserved (Eneqvist et al., 2004; Folli et al., 2003). The thyroid hormone binding site is also highly conserved, although Ser 117 (human sequence) is substituted by Thr in sea bream TTR (Folli et al., 2003). This substitution however is unlikely to affect TH binding according to the predicted structure of TTR in complex with L-thyroxine (Wojtczak et al., 2001). Despite conservation of TTR structure between sea bream and other vertebrates, the surface potential, most noticeably in the thyroid hormone-binding site is more negative in the sea bream and the N-terminus of the protein is longer (Power et al., 2000a).

As a first step to understanding the basis for the differing hormone affinities between fish and mammalian TTR, wild type and N-terminal mutant sea bream TTRs were produced. Ligand-binding studies were carried out in order to characterise the TH:TTR interactions. Also, the consequences of the N-terminal mutations for acid-mediated TTR fibril formation were assessed. Such studies may help to elucidate the regions of the molecule which are of importance in TTR TH binding preference and tetramer stability.

Materials and Methods

Cloning of sea bream TTRWT and mutants

A construct corresponding to mature sea bream TTRWT (sbTTRWT) or TTR mutants in which the first 12 (sbTTRM12) or first 6 N-terminal amino acids (sbTTRM6) were omitted, were amplified by PCR using the full-length sea bream TTR cDNA in pBuescript SK(+) as the template (Santos and Power, 1999). sbrTTRWT vector pET 24d(+) "construction" was supplied by Therese Eneqvist (Umea University) containing the protein encoding gene (lacking the signal sequence). Recombinant sbTTRWT (sbrTTRWT) and mutants proteins

were produced by PCR with specific primers (table 1). The primers used to amplify mutant TTRs were designed to introduce a restriction enzyme site 5' *NheI* flanking the vector's N-terminal methionine and a *BamH I* 3'cleavage site following the C-terminal stop codon. PCR reaction was as described in chapter 2 in a final 25 µl reaction volume. Thermocycle used was of 94 ºC for 3 min, followed by 35 cycles of 94 ºC for 40 s (denaturing), 69 or 67 ºC (optimal annealing temperature determined for sbrTTRM6 and M12 respectively) for 60 s and 72 ºC for 60 s followed by a elongation step of 5 min at 72 ºC. The PCR reaction products were firstly subcloned into pGEM-T Easy vector (Promega, VWR, Portugal) and transformed into XL1B *E. coli* for easier manipulation to achieve the desired mutation (see chapter 2). Final insert sequence was ligated using T_4 DNA ligase Ready-To-Go kit (Amersham Biosciences, UK) into pET 24b(+) vector (Novagen, Germany) which had been cut with the appropriate restriction enzymes to generate compatible ends (see chapter 2). The recombinant vector was used to transform (Hanahan, 1983) *E. coli* BL21DE3 pLySs (Novagen) which were then plated on LB agar plates containing 30 $\mu g.ml^{-1}$ kanamycin and 34 $\mu g.ml^{-1}$ cloramphenicol. Plasmid DNA was isolated from the bacterial culture using the alkaline lysis method (Birnboim and Doly, 1979) and the appropriateness of constructs confirmed by restriction digest and DNA sequencing (Macrogen, Korea).

Table 1. Primers used for amplification of sbrTTRWT, M12 and M6.

	Primers (5'→ 3')	
	Forward	Reverse
sbrTTRWT	TTT TTC ATG ACC CCC ACC CCC ACG	TTT CGA GCT CAC TCG TGT ACG CTG GAG
sbrTTRM12	**GCT AGC** TGT CCT CTG ATG GTA AAA ATC C	<u>GGA TCC</u> TCA CTC GTG TAC GCT GGA GAC
sbrTTRM6	**GCT AGC** GGA GGC TCA GAC ACC AGG TG	<u>GGA TCC</u> TCA CTC GTG TAC GCT GGA GAC

Bold and underlined sequences represent the restriction enzyme sites for *Nhe I* and *BamHI*, respectively, introduced for correct insertion in the expression vector.

Production of sea bream recombinant TTRWT and mutants

Recombinant protein expression was carried out in *E. coli* strain BL21DE3pLysS transformed with the appropriate expression vector constructs (pET24d(+)/sbTTRWT, pET24b(+)/sbTTRM6 or pET24b(+)/sbTTRM12) as described in chapter 2. After expression, the protein extract (~8 ml) containing wild type or mutant sbrTTRs was purified in native conditions using the preparative continuous-elution electrophoresis system (Model 491Prep Cell, Bio-Rad laboratories) and further concentrated, dialyzed and quantified as described in detail in chapter 2. Purified sbrTTRWT was used to raise a polyclonal sbTTR antibody and the proteins were analysed by SDS-PAGE, native PAGE and western blot using colorimetric detection as described in chapter 2.

[^{125}I]-T_3 binding to sbrTTRWT and mutants by native PAGE

In order to analyze the thyroid hormone binding ability of sbrTTRWT, M6 and M12, the purified proteins were incubated on ice for 1 h with [^{125}I]-T_3, as T_3 was previously indicated to strongly bind fish TTR (Santos and Power, 1999; Yamauchi et al., 1999). A parallel reaction with [^{125}I]-T_4 (0.01 µCi) and a human plasma (1.5µl) sample which contain albumin (67 kDa) that binds T_4 was also conducted. The samples were then separated by electrophoresis on a 12.5 % native glycine-acetate gel at 60 mA in glycine-acetate 1 x running buffer (see appendix). Samples contained 25 µl protein, plus 5 µl of 50 % glycerol and 5 µl of glycine-acetate 5 x glycine-acetate running buffer (see appendix) and finally 1 µl of labeled hormone (0.01 µCi). The resulting gel was dried under vacuum for one hour at 60-80 ºC (Drygel.jr se540, Hoeffer Scientific Instruments, UK) and exposed for 48 h at -80 ºC to Kodak BioMax MS film in a cassette containing an intensifying screen.

THs binding to sbrTTR WT and mutants by [^{125}I]-T_3 binding assay

sbrTTR binding assays were developed using the wild type protein as described in chapter 2. In order to titrate protein concentration, several concentrations of sbrTTRWT (0.05, 0.15, 0.25 and 0.5 µg) were tested. Binding to TTRs was analysed by competition binding assay as described in chapter 2 followed by scatchard analysis to determine the dissociation constants of T_3 and T_4 binding to sbrTTRWT, M6 and M12. Scatchard plots were derived form displacement curves using the softwear Kell-Radlig (Biosoft, UK). The existence of significant differences in the IC_{50} and Kd values obtained was determined by One Way Analysis of Variance (ANOVA) and multiple comparison procedures Tukey (HSD) test. Differences were considered statistically significant at $p<0.05$. All the statistical analysis was performed using Sigma Stat software version 3 (SPSS).

SbrTTRWT and mutants fibril formation under acidic conditions

Production of fibrils by sbrTTR was assessed at several different pH values: pH 7 (control), pH 5.5, pH 4.5 and pH 3.5 using previously described methods (Colon and Kelly, 1992; Hammarstrom et al., 2001; Lai et al., 1996). Briefly, sbrTTR samples (WT and mutants) were diluted to a concentration of 0.1-0.2 mg/ml which is close to the mammalian physiological average in blood (0.2mg/ml). Acetate buffers used for dilution (NaOAC 50 mM, KCl 100 mM) were prepared and NaOH or HCl used to obtain the desired pH. Samples were incubated at 37 ºC for 72h and fibrils were collected by centrifugation at 14000 g for 30 min. The resulting pellet was carefully resuspended in 1 ml of a buffer solution with Thioflavine T (ThT) (Tris 50 mM, 100 mM KCl, ThT 10 µM) prepared immediately before use. To quantify the fibril aggregates in solution, fluorescence measurements were recorded in a Fluoromax 3 spectrofluorometer (Jobin Yvon, Horiba) using a 1 cm path length quartz cell. Excitation and emission slit width was of 5 nm. The excitation wavelength was set at 450 nm and the

emission spectrum recorded between 450-600 nm. Emission at 482 nm is characteristic of ThT bound to amyloid fibrils and is proportional to the quantity in solution (Naiki et al., 1989). The final spectrum was obtained after subtraction of the ThT baseline spectrum and dividing by the exact concentration of each protein. The existence of significant differences between samples was assessed by Two Way ANOVA and multiple comparisons analysis (Holm-Sidak method) using Sigma Stat software version 3 (SPSS). Differences were considered statistically significant at $p<0.05$.

Results

Expression and purification of sea bream recombinant TTRWT and mutants

Sequencing of TTR expression vector constructs confirmed that sbrTTRWT and the two mutants sbrTTRM6 and sbrTTRM12 were successfully ligated into vectors and were in frame. Optimization studies of recombinant protein expression with sbrTTRWT revealed that a 0.2 mM IPTG concentration and 3 h incubation post-induction at 30 ºC were appropriate conditions. Recombinant protein expression was carried out in *E. coli* BL21DE3pLysS strain and production of protein was analysed using denaturing SDS-PAGE 15% followed by western blotting (not shown). A single immunoreactive protein was identified in bacterial lysates with rabbit sbrTTR antiserum. Bacteria (BL21DE3pLysS) transformed with the expression vector pET 24b(+) without a sbTTRWT insert and vector pET24b(+)/sbTTRWT but without IPTG induction were used as controls and did not express the protein. The same expression conditions were used for production of the mutant proteins sbrTTRM6 and sbrTTRM12. Analysis of purified proteins by denaturing SDS-PAGE (15% polyacrylamide) revealed that the monomeric sbrTTRs were similar to the predicted molecular weight of 12, 13 and 14 KDa for sbrTTRM12, sbrTTRM6 and sbrTTRWT respectively (fig. 1A). The

functional form of TTR is a tetramer and analysis of the purified recombinant proteins was also carried out by native PAGE (10% polyacrylamide) followed by silver staining. Single bands correspondent to sbrTTRM12, M6 and WT were detected (fig.1B) and both monomeric and native WT and mutant TTRs were immunoreactive with sbTTRWT antisera as revealed by western blot (fig. 1C and D respectively).

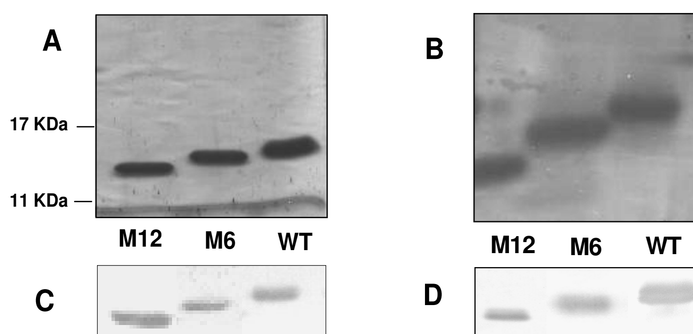

Figure 1. Analysis of purified sbrTTRWT (WT) and mutants sbrTTRM6 (M6) and sbrTTRM12 (M12). **A.** SDS-PAGE 15 % followed by silver staining of purified and concentrated recombinant proteins. Molecular weight markers are indicated. **B**. Analysis of purified and concentrated recombinant protein using Ornstein-Davis 10% native PAGE, followed by silver staining. **C and D**. Western blot analysis of the correspondent protein samples in denaturing and native PAGE is shown below the correspondent figure. The samples were transferred to a PVDF membrane and immunostained using a primary antisera raised in rabbit against sbrTTRWT (1:10000) and a secondary antisera against rabbit Ig conjugated to PAP (1:5000). Colour development was carried out using 3,3'-diaminobenzidine (DAB).

Analysis of sbrTTRWT and mutants binding to [^{125} I]-T_3 by native PAGE

The native PAGE autoradiographic patterns after 48 h exposure are shown on fig. 2. After pre-incubation of TTR with labeled [^{125}I]-T_3, different gel migration patterns were detected for the labeled hormone. [^{125}I]-T_3 alone migrated close to the electrophoretic front, [^{125}I]-T_3 bound to WT and both mutant TTRs had a retarded migration and migrated in a position similar to the one found for the proteins in the native gel (fig.1). The results indicate that wild type and both mutant TTRs bind [^{125}I]-T_3 in the solid phase assay.

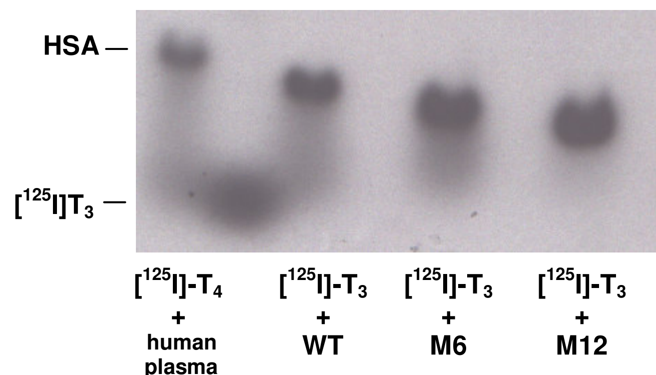

Figure 2. Analysis of [^{125}I]-T_3 binding to sbrTTR by native glycine-acetate gel electrophoresis. Purified recombinant sbrTTRWT, M6 and M12 were incubated at 0 ºC for 2 h with labeled [^{125}I]-T_3 before run on the gel. The gel was dried and autoradiographed with 48 h exposure at -80 ºC. As indicated, first lane shows human plasma incubated with [^{125}I]-T_4 and human serum albumin (HSA) is indicated; Second lane shows [^{125}I]-T_3 pattern in the absence of TTR; the other lanes, as indicated, correspond to sbrTTRWT, M6 an M12 incubated with [^{125}I]-T_3.

THs binding to sbrTTRWT and mutants by [^{125}I]-T_3 binding assay

In order to establish the optimal concentration of protein for sbrTTR binding assays, four different concentrations of sbrTTRWT were tested. [^{125}I]T_3 binding was found to increase up to a protein concentration of 0.10 µg/tube, after which only a modest increase was observed (fig. 3A). A concentration of recombinant protein of 0.05 µg/tube was found efficient and was used thereafter for all binding assays with wild type and mutant sbrTTR. The competitive binding assays revealed that [^{125}I]-T_3 binding to sbrTTRWT was displaced both by increasing unlabeled T_3 and T_4 with similar IC_{50} values (fig. 3B and table 2). Also, Kd values found for both thyroid hormones (fig. 4A and B and table 2) binding to sbrTTRWT were not significantly different ($p>0.05$). These results suggest sbTTRWT has a similar binding affinity for T_3 and T_4, in contrast to previous studies with piscine TTR which suggest it has a higher affinity for T_3 than T_4 (Santos and Power, 1999; Yamauchi et al., 1999).

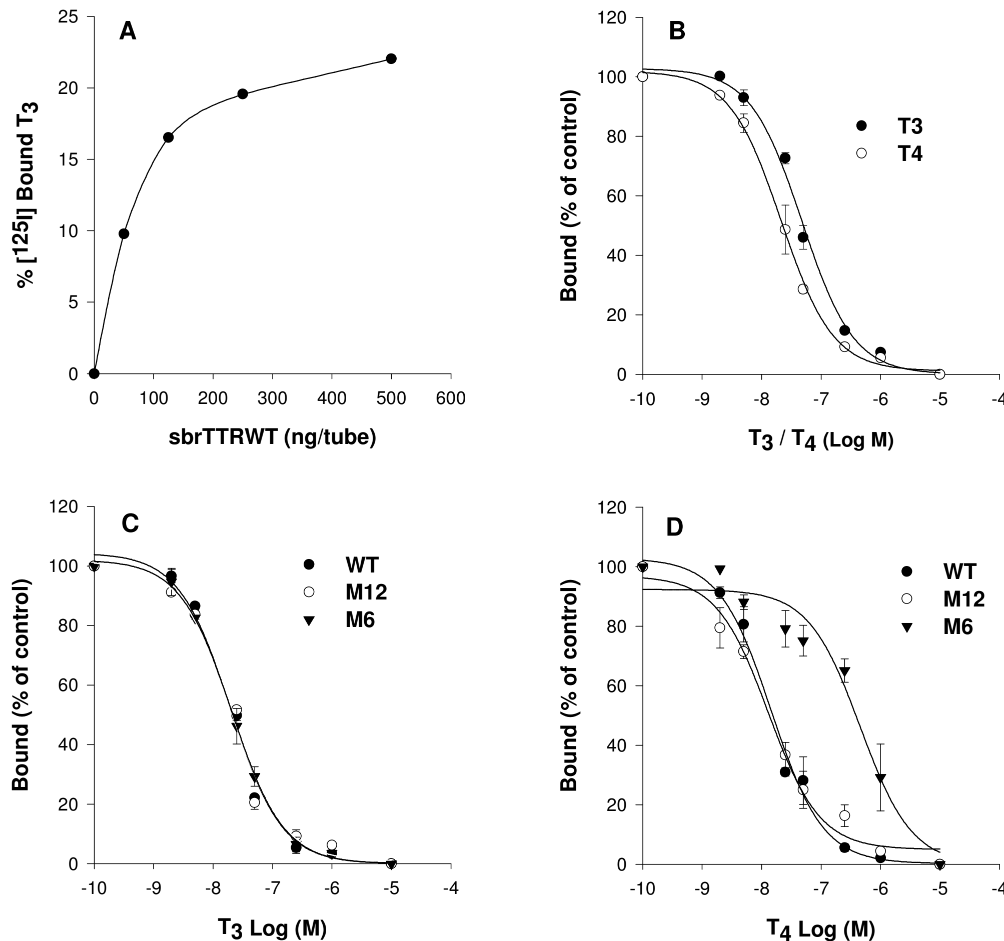

Figure 3. Ligand binding studies of sbrTTRWT (WT), sbrTTRM12 (M12) and sbrTTRM6 (M6). All data represented are the mean±SE of duplicate determinations and each assay was independently repeated three or more times. **A**. Purified sbrTTRWT was incubated at several different concentrations (0-0.5µg) with 0.1nM [^{125}I] T_3 in the absence or presence of 10 µM unlabeled T_3 (for determination of non-specific binding) at 0ºC for 2 h to determine the best working concentartion. **B**. Competitive inhibition of [^{125}I]T_3 binding to sbrTTRWT at 50ng/200 µL final volume by increasing concentrations of unlabeled T_3 and T_4 (0-10 µM). **C**. Competitive inhibition of [^{125}I]T_3 binding to sbrTTRWT, M12 and M6 by increasing concentrations of cold T_3 ranging from 0 to 10µM. **D**. Competitive inhibition of [^{125}I]T_3 binding to sbrTTRWT, M12 and M6 by increasing concentrations of cold T_4 ranging from 0 to 10 µM. Data for competition assays is represented as percentage of control (absence of competitor).

In order to test the effect of N-terminal deletions on the binding affinity of sbrTTR to THs, parallel competition binding experiments were performed with the three proteins, sbrTTRWT, sbrTTRM6 and sbrTTRM12 as previously described (see methods). Binding of [^{125}I]-T_3 to

WT and mutant sbrTTRs was inhibited by unlabeled T_3 in a similar way for each of the proteins tested as shown by the almost overlapping competition curves and close IC_{50} values (fig. 3C and table 2). This result seems to suggest that truncation of the N-terminal region of sbrTTR has no major influence on T_3 binding affinity. Specific binding of the proteins to T_4 was also determined and no significant difference was found in the IC_{50} value of both in sbrTTRWT and sbrTTRM12 (fig. 3D and table 2). However, a significantly ($p<0.001$) higher IC_{50} value (622 ± 98.9 nM) was obtained for T_4 binding to the mutant sbrTTRM6 when compared to sbrTTRWT and sbrTTRM12. In agreement with the preceding results, Scatchard analysis revealed close Kd values for sbrTTRWT and sbrTTRM12 binding to both T_3 and T_4 and for sbrTTRM6 binding to T_3 (see fig. 4 C, D, E and table 2). However, again a significantly ($p<0.001$) higher dissociation constant of 252.3 ± 15.8 was found for sbrTTRM6 binding to the hormone T_4.

Table 2. Summary of IC_{50} and Kd values for sbrTTRWT, M12 and M6 binding to THs. Values are presented as mean ± SE for the sbrTTRWT (n = 7 for IC_{50} and n=5 for Kd) and mutants (n = 3). One way ANOVA and multiple comparison Tukey test were used for comparison of all values for each parameter determined (IC_{50} or Kd). Only T_4 binding to mutant sbrTTRM6 was found to be different either between IC_{50} or Kd values (*$p<0.001$).

	IC_{50} values			**Kd values**		
	sbrTTRWT	sbrTTRM12	sbrTTRM6	sbrTTRWT	sbrTTRM12	sbrTTRM6
T_3	19.6±3,3nM	17.9±3.2 nM	21±1.9 nM	10.6±1.7nM	13.5±3,4M	8.47 ± 0.4 nM
T_4	18.3±1.5nM	21.8±4.0 nM	622*±98.9nM	9.8 ±0.97nM	17.9±4,2M	252.3*±15,8nM

Overall the results seem to indicate that N-terminal truncation does not affect the affinity of sbrTTR for triiodothyronine (T_3). Although the same cannot be said about its binding to thyroxin (T_4), in which intriguingly, removal of the first 6 N-terminal amino acids of sbrTTR

caused a great loss of affinity (approximately 25 x lower), while removal of the first 12 amino acids caused no significant change ($p>0.05$) in binding affinity compared to sbTTRWT.

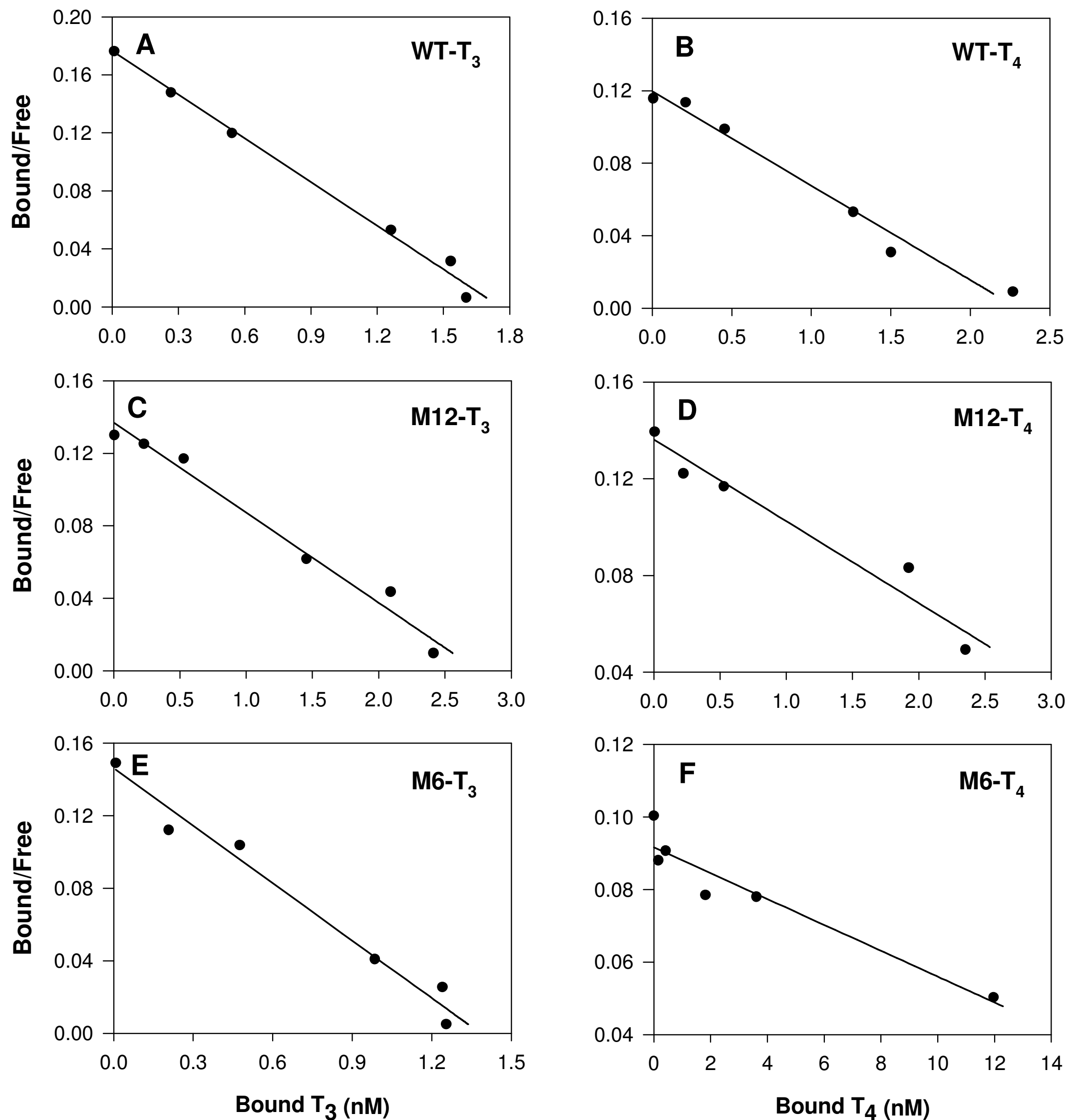

Figure 4. Scatchard analysis of sbrTTRWT and mutants binding to THs. Plots represent sbrTTRWT (WT), sbrTTRM12 (M12) and sbrTTRM6 (M6) binding to T_3 (**A**, **C** and **E**) and T_4 (**B**, **D** and **F**) respectively. Each plot was estimated from displacement curves using the softwear Kell-Radlig (Biosoft) and Kd values determined are the mean of three or more independent determinations carried out in duplicate in each assay.

Amyloid fibril formation by sea bream recombinant TTRWT and mutants

Human TTR WT was previously shown to form fibrils after prolonged incubation at 37 ºC and low pH (Colon and Kelly, 1992). In the present study sbrTTRWT and N-terminal mutants were analysed for their ability to form fibrils in acidic conditions. The amount of fibril formation at increasingly acidic pH was monitored by fluorescence measurements in the presence of the dye Thioflavine T. As emission at 482 nm is characteristic of ThT bound to fibrils, fluorescence spectrum were recorded between 450-600 nm. The maximum emission intensity found for each protein at each pH was related to that obtained for the sbrTTRWT at pH 3.5 (maximum value found) which was taken as 100%. The final result is depicted in fig. 5 as a bar graph and shows that sbrTTRWT, in common with human TTR, also has the ability to form fibrils in acidic conditions *in vitro*. However, the extent of fibril formation only increases dramatically at pH 3.5 ($p<0.001$ when compared to pH 7), whereas for the human protein, maximum fibrillation was found to occur at pH 4.4. As for the mutant proteins, sbrTTRM12 showed significant amyloidogenic properties at an even higher pH of 4.5 with no further increase at pH3.5. Interestingly, for the mutant sbrTTRM6 no significant acid-induced fibril increase was found ($p>0.05$) even at pH 3.5 ($p<0.001$) when compared to the WT at the same pH. sbrTTRM6 appears to have lost its amyloidogenic properties in acidic conditions. The result seem to indicate that removal of the first 12 N-terminal amino acids slightly decreases sbTTR stability, as fibrils start to form at less acidic conditions when compared to the WT (pH 4.5). However, removal of only the first 6 N-terminal amino acids seems to completely inhibit amyloid fibril formation *in vitro* under acidic conditions.

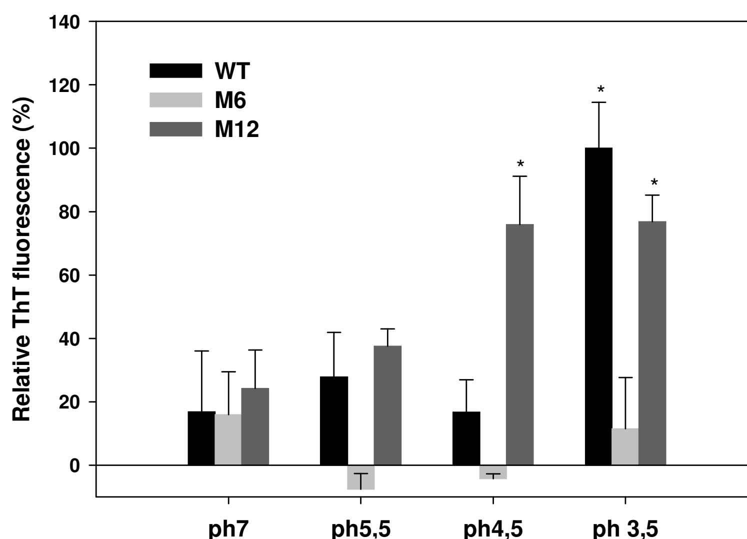

Figure 5. Comparison of sbrTTRWT, M6 and M12 fibril formation after partial acid denaturation at the indicated pHs. Fibril production at each pH was determined by fluorescence emission at 482nm in the presence of the dye Thioflavine T. The highest emission intensity obtained for sbrTTRWT at pH 3.5 was considered as 100% and all the other measurements were determined relative to it. Multiple comparison statistical analysis between all the samples revealed significant (*) increase in fibril formation only for sbrTTRWT at pH 3.5 and at both pH 4.5 and 3.5 for the mutant M12. As for the mutant M6 no significant increase in fibril formation was found between the different pHs. At pH3.5 and pH4.5, M6 ability to produce fibrils was residual when compared to the WT ($p<0.001$) and M12 ($p<0.001$) respectively.

Discussion

Evolution of TTR binding affinity for THs

Over the last few years, TTR cDNA and amino acids sequences have been described for a number of representatives of different phylogenetic groups. Several studies concerning TTR affinity for the thyroid hormones T_3 and T_4 seem to indicate that during evolution TTR progressively lost its higher affinity for T_3 in lower vertebrates and became a T_4 binder in eutherian mammals, (reviewed in Power et al. (2000a)). This evolution is consistent with physiologically advantageous adaptations regarding THs transport in which T_4 acts as a precursor hormone liberated from the thyroid gland and conversion to T_3 occurs in a tissue-

specific manner being regulated by deiodinases. Also, the strong affinity of TTR for T_4 counteracts the easy permeation of THs into the intracellular compartment as a consequence of their lypophilic character. This notion has gained support from studies of T_3 and T_4 affinity for TTR in reptiles (Achen et al., 1993; Prapunpoj et al., 2002), amphibians and fish (Eneqvist et al., 2003; Santos and Power, 1999; Yamauchi et al., 1993; Yamauchi et al., 1999). However, values reported so far to describe affinities between TTRs and THs are very variable between studies even for the same proteins, probably as a consequence of assay methods and protein origin (serum or recombinant). For instance, Eneqvist, Lundberg *et al* in 2003 (Eneqvist et al., 2003) report that sea bream recombinant TTR has a stronger affinity for T_3 in a dot-blot analysis, although in the same assay no binding of T_3 to human recombinant TTR was detected. This may suggest that TH binding to recombinant TTRs may be weaker than native TTR in serum. Also the characteristics of these solid-phase methods which involve protein binding to membranes may affect protein conformation and in this way TH binding. Sea bream recombinant TTR, has been indicated to bind preferentially T_3 but only on qualitative solid phase gel assays (Santos and Power, 1999). In other fish species, values for TTR:TH affinity have been reported for masu salmon serum TTR (Yamauchi et al., 1999) which had 3 times higher affinity for T_3 than T_4. In the present study, in contrast with previous studies, we report that sbTTR has similar affinity for T_3 and T_4 in a liquid phase competitive binding assay.

Comparison of TTR sequences from representative species reveals a high degree of conservation between eutherians, marsupials, birds and lizards. However, sequence identity of TTR from those species and amphibian and fish is lower (Power et al., 2000a). Also, comparative studies of the three-dimensional structure determined for human, rat, chicken and sea bream TTR, revealed a highly conserved tetrameric structure but a surface potential, especially at the TH binding site, much more negative in chicken and sea bream (Power et al.,

2000a). Moreover, the difference in affinity of T_3 and T_4 binding to masu salmon TTR, was not as great as observed with other species, such as bullfrog where a 360 times greater affinity was found for T_3 than T_4 (Yamauchi et al., 1993). Such observations taken together with the data presented here for sbrTTR, suggest that fish TTR has a similar affinity for both THs. It would appear that although from amphibians onwards TTR evolved from a T_3 to a T_4 binder by a Neo-Darwinism mechanism of evolution, in fish an alternative evolutionary process may have occurred. Nonetheless further studies of TTR:TH binding are needed from representative of the main classes of fish which are the most numerous and diverse vertebrates (Helfman et al., 1997). Moreover, studies of ancient species (hagfish and lamprey) close to the divergence of the fishes from the lineage giving rise to tetrapods will be necessary to establish the TH binding characteristics of TTR in common ancestral vertebrates. The differing hormone affinity of TTR in modern teleosts compared to other vertebrates raises interesting questions about the functional significance of this change.

Role of the N-terminal region on sea bream TTR binding

Multiple amino acid sequence alignments of monomeric TTR from several species show a high degree of conservation for this protein. More specifically, it reveals that the amino acids predicted to be involved in the TH binding channel are almost 100% conserved during evolution, suggesting that TH binding is probably the most important function of the protein. However the N-terminal region of the polypeptide seems to be the most variable region showing progressive changes in length and hydropathy from fish to mammals. This segment is shorter and more hydrophilic in eutherians becoming longer and more hydrophobic in marsupials (with two additional amino acids), and in birds, reptile and amphibian (with three additional amino acids). These changes could be explained by splice site shifts of intron 1 as previously proposed (Aldred et al., 1997), but their effect on protein function is not yet clear.

A shorter and more hydrophilic tail seems to be coincident with an increase in affinity for T_4. Moreover, the fact that in the tetrameric structure, this region is thought to be situated at the entrance of the binding channel suggests that its size could influence TH access to the binding sites. These observations led to the hypothesis that the length and hydrophilic nature of the N-terminus are important for TH binding properties of TTR.

So far, binding studies involving recombinant chimeric xenopus/crocodile TTR and recently with human/crocodile TTR provided experimental evidence that this region influences TH binding affinity of TTR (Prapunpoj et al., 2006; Prapunpoj et al., 2002). In order to shed more light on this matter, in the present study, two mutated sbrTTRs were produced, in which the first 6 (sbrTTRM6) and 12 (sbrTTRM12) amino acids were removed from the N-terminal region of the protein. The sbrTTRM12 mutant maintained TH binding characteristics close to sbrTTRWT, while the sbrTTRM6 mutant had approximately 25 times lower affinity for T_4 but not for T_3. This curious result could indicate that the size of the N-terminus is not directly related to the access of THs to the binding site as has been suggested (Power et al., 2000a) but that hormone binding is probably most affected by the properties of the amino acids in this region. This influence could be due directly to the amino acids located at the extremity of the molecule, or because of the overall charge or conformation acquired by this region and possible interactions established with the binding channel.

Analysis of the hydrophobicity profile of sbTTR N-terminal sequence using Protscale tool at http://www.expasy.ch/tools/protscale.html and according to the amino acid scale by Kyte & Doolittle shows that hydrophobicity increases towards the inside of the N-terminal sequence of sbTTR monomer. Therefore, the N-terminus of sbrTTRM12 is more hydrophobic than in sbrTTRM6 and even more than sbrTTRWT. In light of these observations our results do not support the notion that increased hydrophilicity is associated with a higher affinity for T_4 and suggest that other factors must be taken in considerations when analysing the binding

properties of TTR. Nonetheless the N-terminal region does appear to be important for determining hormone affinity possibly through its interaction with the binding channel. In the electron density map of sbTTR, unidentified electron density was found in the centre of the channel which could correspond to N-terminal residues of the protein (Eneqvist et al., 2004). Structural studies also show that there are several amino acid substitutions in the hydrophobic core of piscine TTR compared to the human protein and that residue Val14 (close to the N-terminus) can affect the position of the otherwise conserved residues. Furthermore, Lys15, seems to play a crucial role in hormone binding to the protein. Together with Glu54, this residue is located at the entrance of the channel and interacts strongly with THs (Eneqvist et al., 2004). Such evidence suggests that amino acids located close to N-terminus can strongly influence the surrounding environment and thus affect hormone binding to the protein. Therefore it is not surprising that some amino acid modifications can cause strong affinity differences while at the same time other modifications have no effect. In conclusion, the results of the present study indicate that the N-terminal region plays an important role on the ability of TTR to bind THs. However, the results do not necessarily correlate with the binding evolution to T_4, or with the size of the N-terminus but with an overall combination of specific amino acid properties.

N-terminal region and sea bream recombinantTTR tetramer stability

It is known that human TTR can form amyloid fibrils in acidic conditions. *In vitro* studies have shown that TTR forms fibrils upon incubation at low pH at 37 ºC (Colon and Kelly, 1992). However, at physiological concentrations and pH it retains its native structure. TTR amyloid begins to form only when tetramers dissociate into their monomeric intermediates that self assemble into fibrils (Damas et al., 1996; Hammarstrom et al., 2002; Lai et al., 1996; Quintas et al., 1999). Thus, the consensual approach to prevent amylodogenesis relies on

stabilization of the tetrameric native conformation. Several studies have shown that the binding of many small molecules including the natural ligand T_4 to TTR's central channel favours tetramer fold stability reducing fibril formation (Adamski-Werner et al., 2004; Klabunde et al., 2000; Miroy et al., 1996; Morais-de-Sa et al., 2004; Purkey et al., 2001). So far, no particular region of the TTR molecule has been associated with its amyloidogenic properties, although previous findings strongly relate fibril formation with the central thyroid hormone-binding channel stability. In the present study we suggest that changes in the N-terminal region of the protein monomer can affect sbTTR affinity for THs by interfering with the properties of the binding channel. In this way, such changes might also affect tetramer stability and consequent fibril formation.

Fluorimetric measurements were used to compare the extent of fibril formation at low pH for sbrTTRWT and the two N-terminal mutants sbrTTRM12 and sbrTTRM6. Interestingly, the mutant sbrTTRM6 that lost affinity for the hormone T_4 seemed to be more stable and did not form fibrils at a pH as low as 3.5. Again, charge or conformational changes imposed by N-terminal amino acids are likely to be involved. One could speculate that the electrostatic or structural characteristics of sbrTTRM6 N-terminal residues may, by interacting with the binding region, strengthen the tetramer stability preventing its dissociation and amyloidosis. The greater resistance of sbrTTR to fibril formation found in the present study compared to previous studies of the human protein (Eneqvist et al., 2003; Lai et al., 1996) makes further comparative analysis concerning TTR stability of great interest. Moreover, the possibility of relating a specific region with protein fibril formation may offer a means by which to combat this process, and it will be of importance to investigate this further.

Acknowledgments

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Regulation of transthyretin by thyroid hormones in fish

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Abstract

Transthyretin (TTR) is a thyroid hormone binding protein (THBP) which in its tetrameric form transports thyroid hormones (THs), thyroxine (T_4) and triiodothyronine (T_3) in the blood of vertebrates. The principal site of production of TTR is the liver but in the sea bream TTR mRNA is also present in the heart, intestine and brain. The regulation of TTR is unstudied in fish and the normal circulating level of this THBP is unknown. The aim of the present study was to establish factors which regulate TTR production in fish. As a first step a number of tools were generated; sea bream recombinant TTR (sbrTTR) and specific sbrTTR antisera which were used to establish an ELISA (enzyme-linked immunosorbent assay) for measuring TTR plasma levels. Subsequently, an experiment was conducted to determine the influence of THs on TTR production. Circulating physiological levels of TTR in sea bream determined by ELISA are approximately 3.8 $\mu g.ml^{-1}$. Administration of T_3 and T_4 to sea bream significantly increased ($p<0.001$ and $p<0.005$, respectively) the concentration of circulating TTR (~11.5 $\mu g.ml^{-1}$) in relation to control fish, but did not change gene transcription in the liver. Methimazol an antithyroid agent, failed to significantly reduce circulating TH below control levels but significantly increased ($p<0.005$) plasma TTR levels (approximately 10.8 $\mu g.ml^{-1}$) and decreased ($p<0.05$) transcription in the liver. Future studies will aim to elucidate in more detail these regulatory pathways.

Introduction

Transthyretin (TTR) is one of the binding proteins that convey thyroid hormones (THs) in the bloodstream. This role is shared with a number of other proteins and lipoproteins, although thyroxine binding globulin (TBG), and serum albumin are the other major serum proteins implicated in vertebrates (Bartalena, 1990; Langsteger, 1997; Schussler, 2000). In humans, TTR plasma concentrations (~300 mg/l) largely exceeds that of total THs (0.05-0.12 mg/l) and less than 1% is involved in hormone transport raising questions about other potential functions (Robbins J., 1986). In vertebrates, TTR also plays an important role in retinol transport as it also binds retinol-binding protein (RBP) (Kanai et al., 1968; Peterson, 1971; van Jaarsveld et al., 1973) and both proteins are secreted by the liver in the same ratio remaining closely bound in plasma (Ingenbleek and Bernstein, 1999). Although TTR is principally a secretory product of the liver (Schreiber, 1987) it is also highly expressed in the choroid plexus of the brain in some vertebrates (Harms et al., 1991) and this has led to the formulation of several models for its evolution.

In mammals, TTR is an acute phase protein and serum levels are abruptly depressed in response to inflammation or infection and it has also been extensively characterised as a marker of malnutrition or stress (Brugler et al., 2002; Ingenbleek and Bernstein, 1999; Ingenbleek et al., 1972; Ingenbleek and Young, 1994; Ingenbleek and Young, 2002). In fact although the response of TTR to a number of different physiological or experimental conditions has been characterised, relatively little is known about the way in which endocrine factors and in particular, THs, regulate TTR synthesis. Studies using thyroidectomized rats, suggest low susceptibility of TTR to thyroid status and point to the involvement of regulatory pituitary factors on TTR´s production (Franklyn et al., 1989; Vranckx et al., 1994). Similar studies involving other vertebrates are scarce or non-existent.

TTR has been identified in teleost fish and shown to bind THs (Kawakami et al., 2006; Santos and Power, 1999; Yamauchi et al., 1999) suggesting it also behaves as a thyroid hormone binding protein (THBP) in these vertebrates, although its function as a RBP binder, remains unclear (Berni et al., 1992; Folli et al., 2003; Zapponi et al., 1992). Physiological factors which affect TTR production in fish are largely unstudied, although estradiol (Funkenstein et al., 2000) and food restriction (Power et al., 2000b) down regulate liver TTR mRNA expression. An increase in E2 levels during the reproductive cycle is usually coincident with a reduction in TH plasma concentrations and it is also well established that food shortage causes down regulation of the hypothalamus-pituitary-thyroid axis resulting in reduction in circulating THs (Eales and Himick, 1988; Scott-Thomas et al., 1992). Taken together the preceding observations appear to indicate that reduction of TTR gene expression in fish is coincident with situations in which THs are depleted, suggesting THs may directly or indirectly regulate plasma TTR.

The objective of the present study therefore was to establish if TH levels can directly control TTR expression and secretion into the circulation. In this context, sea bream recombinant TTR was produced, specific antisera were generated and an ELISA for measurement of plasma TTR was established. The effect of *in vivo* treatment with T_3, T_4 and Methimazol (MMI, an antithyroid drug) on total plasma protein, TTR hepatic expression and TTR plasma concentrations was assessed in sea bream.

Materials and Methods

Animals and Experiments

Blood samples used for Western blot and establishment of ELISA were collected from "stock" sea bream of different ages (6 months, 1.5 years and 3 years) maintained in 1000 l open sea water circuits at ambient temperature and salinity for the Algarve, Portugal. Fish were fed daily (3-5 % total fish weight) on commercial ration (PROVIMI, Portugal). Blood was removed from the caudal vessel of anaesthetised fish (2-phenoxyethanol, 1:1000 v/v; Sigma-Aldrich) using a heparinised syringe. Samples were immediately centrifuged at 1000 g for 10 min at 4 ºC and plasma separated and frozen in liquid nitrogen.

Twenty four sea bream were weighed (mean ± SD: 43.43 g ± 7.89 g) and the length measured (Mean ± SD: 13.61 cm ± 0.70 cm) and then randomly assigned to four groups representing three different treatments and the control; each group contained six fish. Fish were maintained in conical tanks of 250 l with a through flow (250 l/h) of well-aerated seawater under natural photoperiod (14 light:10 dark), salinity (38.28 ± 0.25 %) and water temperature (24.54 ± 1.45 ºC) for summer in the Algarve. Fish were acclimatised to the experimental tanks for one week prior to the start of the experiments. After this period, MMI and hormones T_4 and T_3 (1 mg/Kg wet weight) were administered by intraperitoneal injection to fish using coconut oil as a vehicle. Control fish received injections of coconut oil alone. All fish were fed with commercial diets at the rate of 3 % of total fish weight per tank per day with commercial dry pellets (sea bream ration, PROVIMI, Portugal), both during the acclimatisation (1 week) and experimental periods (10 days). No mortality was registered during the experiment. At the end of the experiment fish were anaesthetised with 2-phenoxyethanol (1:1000 v/v; Sigma-Aldrich) and blood samples collected as described above. Weight (g) and length (cm) were measured and fish were killed by decapitation and a sample of liver taken and frozen in liquid nitrogen.

Maintenance and manipulation of fish was conducted in accordance with the Guidelines of the European Union Council (86/609/EU).

Production of recombinant TTR from sea bream

Sea bream TTR cDNA (Santos and Power, 1999) subcloned in the vector pET24d(+) (Promega) was used to produce sea bream recombinant TTR (sbrTTR) in competent *E. coli* BL21DE3pLysS by IPTG (0.2 mM) induction and further isolated and concentrated. All these procedures are given in detail in the general methods section (chapter 2).

Production and characterisation of sbrTTR polyclonal antibodies

The pure protein was used to raise polyclonal sbrTTR antibodies (see chapter 2). The obtained antiserum and a pre-immune serum sample were tested by western blot with colorimetric detection as described in chapter 2. Briefly, pure sbrTTR samples were fractionated by SDS-PAGE (15%). Duplicate gels were run and one was stained with Commassie blue and the other was transferred to hydrophobic polyvinylidene difluoride (PVD) membranes fo immunoblotting. Membranes were incubated with three different dilutions (1:1000, 1:5000 and 1:10000 in 1x PBS) of rabbit anti-seabream TTR sera for 2h at room temperature washed and further incubated with secondary antibody (anti-rabbit IgG-peroxidase anti-peroxidase 1:5000) prior to colorimetric detection. The same methodology was used for the detection of TTR in sea bream plasma. Samples of plasma were collected (see Animals and Experiments section) and 8 µl of 1:10 dilutions were loaded on a 15% SDS-PAGE and further transferred for western blot analysis.

Development of sbrTTR ELISA

In order to quantify TTR an indirect antigen competitive ELISA method was established using the sbrTTR and specific antisera. A range of assay optimisation procedures were carried

out including assessment of assay buffers; the coating concentration of TTR (10-100ng/well); titration of primary (1:10000-1:160000) and secondary antisera (1:2000-1:60000) to give a maximal optical density (OD_{490nm}) of 1 and a background of 0.05-0.08 (OD_{490nm}); assay incubation times and washing conditions. ELISA was carried out in triplicate for standard and samples in 96-well plates (Nunc, Apogent, Denmark) using the optimised procedure. In brief, wells were coated overnight at 4ºC with 10ng/200µl/well sbrTTR dissolved in bicarbonate buffer (50 mM $NaHCO_3$ and 50 mM Na_2HCO_3 to give a final pH of 9.6) containing 0.1% sodium azide, washed 2 x 5 min with PBS/Tween-20 (0.1% v/v). Anti-sbrTTR (1:10000 dilution) was added to the plate along with either standard protein (3.125- 400 ng/well) or samples (200 µl/well) and incubated for 2 h at 37ºC. Excess reagent was removed by washing 3 x 10 min with PBS/Tween-20 before adding anti-rabbit IgG Peroxidase conjugate (200 µl/well, 1:4000 dilution, Sigma-Aldrich) and incubating for 1 h at 37ºC. Excess reagents were eliminated by washing 3 x 10 min with PBS/Tween-20. Colour was developed with OPD (o-phenylenediamine dihydrochloride, Sigma-Aldrich, 200 µl/well) for 10 min in the dark at room temperature. The reaction was stopped by adding 50 µl/well 2M H_2SO_4 and absorbance read at 490nm in a microtitre plate reader (Biorad, Benchmark). Standard curve, non-specific binding and maximal binding was determined in all assays. ELISA validation included verification of parallelism between samples and the standard curve, the recovery rate of standards added to the assay and the intra-assay and inter-assay variation. Plasma samples were diluted 1:10 and 1:20 prior to ELISA.

Condition factor

The condition factor (*K*) of the fish before and after the treatments was determined as follows: $100 \times W \times L^{-3}$, where W stands for body weight (g) and L for length (cm).

Total plasma protein

Total protein concentration in plasma samples was determined using the Lowry Method (Lowry et al., 1951), and a bovine serum albumin (BSA) standard curve and measuring absorbance at 745 nm.

Radioimmunoassay (RIA)

Thyroid hormone (T_3 and T_4) plasma concentrations were measured using a specific and reproducible radioimmunoassay (RIA). Briefly, plasma samples were diluted (1/10) in phosphate buffer (0.01 M pH 7.6), heat extracted (1h 30 min at 70 ºC) then centrifuged for 5 min at 4 ºC and 1500 g. The total assay volume was 300 μl and was composed of 100 μl of standard or diluted plasma (15 μl of 1/10 diluted plasma in 85 μl buffer), 100 μl of anti T_4 sera (1:8000, ~3 % cross-reactivity with T_3; T2652 - Sigma-Aldrich, Madrid, Spain) or anti T_3 sera (1:15000, <0.01% cross-reactivity with T_4; T2777 Sigma-Aldrich) and 100 μl of tracer ([^{125}I]-T_4 and [^{125}I]-T_3, respectively). Assays were incubated overnight at 4 ºC 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 μ1 of an ice-cold PEG solution (7.5 % w/v PEG 8000; Merck) in assay buffer was added, and the tubes were centrifuged for 10 min, 1500 g at 4 ºC. The supernatants were decanted, and the pellets were counted in a gamma counter (Wizard, Pharmacia-LKB).

Northern blot

Total RNA was extracted from livers using TRI reagent (Sigma-Aldrich) and following the protocol provided. The quantity and quality of extracted RNA was assessed by spectroscopy

(Pharmacia Biotech Ultraspec 3000) by determining absorbance at A_{260}nm and the ratio, A_{260}/A_{280}, respectively.

Seabream TTR and β-actin probes were produced by PCR using specific primers (TTR *forward primer:* 5' AAT CCT GGA CGC AGT GAA AGG G 3'; *reverse primer:* 5' AGC AGC AGA GCC AAG GTG TAG TGA 3'; β-actin *forward primer:* 5' GGC CGC GAC CTC ACA GAC TAC 3'; *reverse primer:* 5' ACC GAG GAA GGA TGG CTG GAA 3') and the recombinant plasmids as template (Santos & Power, 1999; Santos et al, 1997). The thermocycle consisted of 3 min at 94 ºC, followed by 30 cycles of 94 ºC for 1 min; 60 ºC for 1 min and 72 ºC for 1 min (TTR) or 45 s (β-actin) and a final 5 min at 72 ºC. PCR probes were isolated using GFX PCR DNA and Gel Band Purification Kit (Amersham Biotech). These probes were labelled with [α-^{32}P]dCTP (Redivue [^{32}P]dCTP, Amersham Biotech) using a Random Prime Labelling System (Amersham Biotech). Equivalent quantities of liver total RNA (30 µg) were denatured and fractionated on a 5.5% formaldehyde/1.5% agarose gel, using 1x MOPS (Sigma) as running buffer. Fractionated RNA was then transferred to nylon membranes (Amersham Hybond-XL) by capillary transfer, using 10x SSC as transfer buffer and fixed by UV crosslinking. Membranes were pre-hybridized for 2 hours in Church-Gilbert solution at 60ºC and then hybridized overnight in Church Gilbert solution containing sea bream [α-^{32}P]dCTP labelled TTR or β-actin. Subsequently, high stringency washes were carried out and membranes were exposed to intensifying screens (Bio-Rad) for 3 hours and analysed using a phosphoimager (FX Pro Plus System, Bio-Rad). Hybridisation with sea bream β-actin served to normalize RNA loaded in each sample.

Statistics

All data was analysed using One-way analysis of variance (ANOVA) followed by Tukey (HSD) multiple comparison test when statistically significant differences were detected

between treatments. All the statistical analysis was performed using Sigma Stat software version 3 (SPSS, Chicago, IL, USA). Differences were considered statistically significant at $p<0.05$.

Results

Production of sbr TTRWT

SbrTTR was successfully produced by IPTG (2 mM) induction of *E. coli* BL21 pLysS containing pET24d-sbTTR. Continuous elution native electrophoresis resulted in isolation of pure sbrTTR as assessed by the presence of a single band of the expected size for the monomer (14 KDa) using analytical SDS-PAGE 15% (Fig. 1A).

Production of sbrTTR polyclonal antibodies and western blot analysis

A specific high titer polyclonal sbrTTR antiserum was successfully produced. The sbrTTR antiserum at a dilution of 1:10 000 was utilized in western blot and cross-reacted with both the monomer and dimer of the sbrTTR (fig. 1B-III). Pre-immune serum included as a negative control failed to detect sbrTTR in western blot (fig. 1B-IV). Western blot was also used to assess whether the specific antiserum (1:10 000 dilution) cross-reacted and detected sea bream TTR present in plasma, an essential characteristic for its use in ELISA. Analysis by SDS-PAGE (fig. 1C) and western blot (fig. 1D) of plasma samples from sea bream at different stages of development, juvenile (6 months), adult (1.5 years) and mature adult (3.5 years) revealed a single highly specific band of an appropriate size for the TTR monomer in all samples (fig. 1D). Although similar protein loading occurred for each of the plasma samples analyzed, TTR immunoreactivity increased with fish age, suggesting the concentration of plasma TTR differs with age. Moreover, analytical electrophoresis under non-denaturing conditions followed by western blot revealed a single band corresponding to the native TTR tetramer (results not shown).

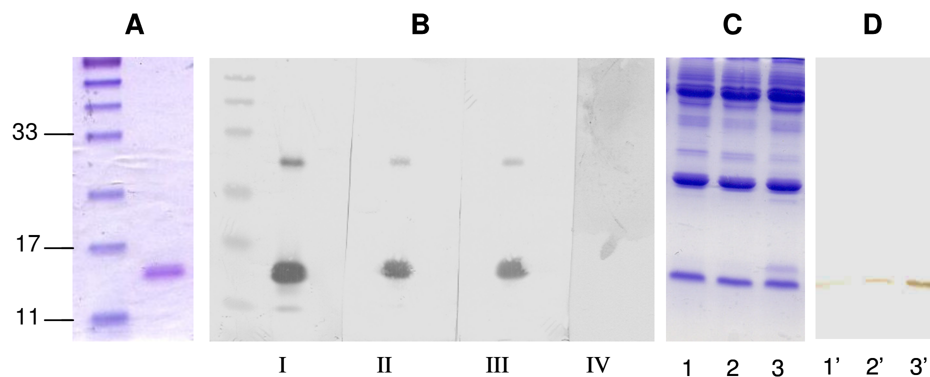


Figure 1. A- SDS-PAGE analysis of purified sbrTTR. **B**- Western blot analysis of sbrTTR using different dilutions of sbrTTR antisera for optimisation, lane I= 1:100, lane II= 1:5000, lane III= 1:10000. Strong bands of the expected size for monomeric (14 KDa) and dimeric (28 KDa) sbrTTR were detected. Lane IV - immunoblot carried out with pre-immune serum failed to detect sbrTTR. **C**- SDS-PAGE and **D**- Western blot analysis of plasma samples from sea bream at different development stages: 1 and 1'- juvenile, 2 and 2'- young adult, 3 and 3'-mature adult. The same protein concentration for each sample was loaded on the gel. Western blot revealed a single band for each sample showing that the anti sbrTTR antisera also cross-reacts with plasma TTR in a specific manner. Band intensity was stronger in older fish, suggesting they have higher concentrations of circulating TTR.

sbrTTR specific ELISA

A simple, reliable and reproducible indirect competitive ELISA was developed using sbrTTR as the standard and a rabbit polyclonal antisera raised against the recombinant protein. The coating concentration of TTR was 10 ng/well and antiserum against sbrTTR was used at a dilution of 1:10 000 which gave a maximal OD_{490nm} of 1 and non-specific background of 0.05-0.08 at OD_{490nm}. This assay had a mean EC_{50} (mid-range of the assay), calculated as the amount of sbrTTR that gives 50 % of the maximal OD of 0.5± 0.015 µg/well (n=4). The lower limit of detection (sensitivity of the assay) was 12.5ng of TTR/well. The sbrTTR ELISA had a negligible cross-reactivity (<0.001 %) with other plasma proteins over the normal working range of the assay 3.125- 400 ng of sbrTTR/well.

The optimal method for fitting the standard curve was the log-logit method, and only the linear region of the curve was used for assessment of sea bream TTR in samples. Linear and logarithmic curve fitting methods were found to either over or under-estimate the concentration of a standard of sbrTTR introduced in the assay.

Sea bream plasma dilution samples gave good parallelism with the standard curve (fig. 2). No non-specific binding effects were found with fish plasma and recovery was 100 ± 19 % (n=9). The inter and intra-assay variance were 3 % (n=4) and 5 % (n=6), respectively. The overall results from the validation studies indicated that the ELISA developed can be used to accurately quantify circulating sea bream TTR.

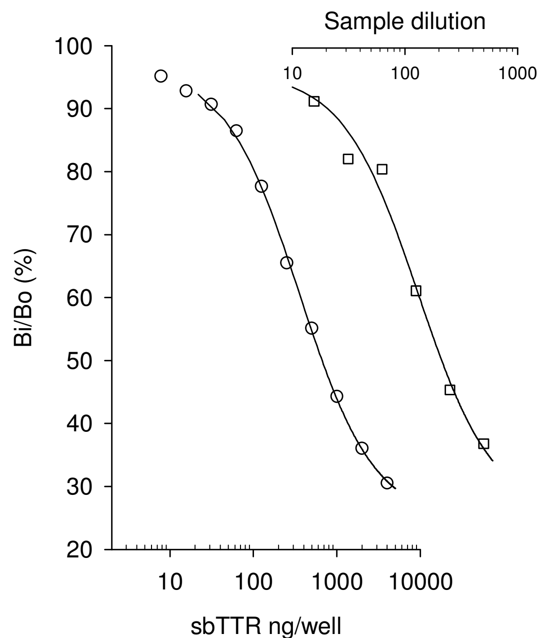


Figure 2. Standard curve of sbrTTR ELISA and dilution curve of plasma sample. Note the dilution curve of plasma is parallel to the standard.

THs and MMI administration

There are few studies available in fish documenting how thyroid hormone implants alter plasma hormone levels, so this was the first factor evaluated to ensure the treatment utilised

(hormone concentration and treatment duration) was adequate. The concentration of plasma T_3 and T_4 in control fish was 35.4 and 36.3 ng/ml respectively, which is in accordance with previously reported values for sea bream (Power et al., 2000b). Fish which received a T_4 implant had a significantly higher ($p<0.001$) plasma T_4 concentration than fish treated with T_3, MMI or control fish (fig. 3A and table 1). In fish treated with T_3 a significant increase ($p<0.01$) in circulating T_4 over control and MMI fish was also found (fig. 3A and table 1) and probably reflects cross-reaction of anti T_4 sera with the elevated plasma T_3 in the samples. T_3 implants were also effective and caused a significant increase ($p<0.001$) in total circulating T_3, over that of control, T_4 or MMI treated fish (fig. 3B and table 1).

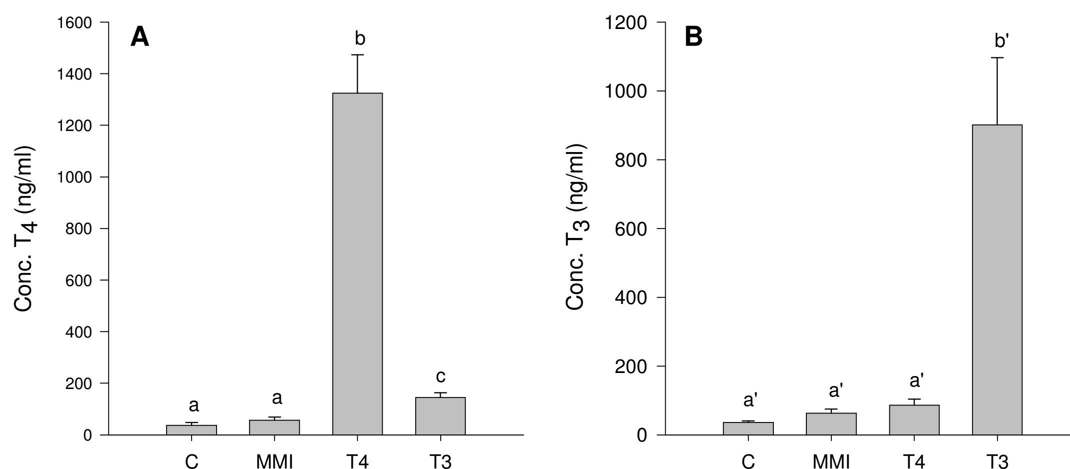


Figure 3. A- Concentration of thyroxine (T_4, ng/ml) measured by RIA in plasma collected from each experimental group of sea bream: C - control implanted with coconut oil alone, MMI - methimazole, T_4 – thyroxine, T_3 - triiodothyronine. Statistical analysis denote a significant increase ($p<0.001$) in plasma T_4 for the T_4 implanted group (b) compared to all other groups. A small but significant increase in T_4 concentration was also verified for the T_3 (c) treated group ($p<0.01$) compared to the control group. **B-** Concentration of triiodothyronine (T_3, ng/ml) in plasma from sea bream implanted with MMI, T_4, T_3 or coconut oil alone (C). T_3 (b') plasma levels were significantly elevated ($p<0.001$) compared to all other groups in the T_3 injected group. Data corresponds to the mean ± SE (n=5 or 6/group).

Treatment with the antithyroid agent MMI, which acts through inhibition of thyroid peroxidase (TPO) in the thyroid follicle in mammals (Engler et al., 1982), was expected to suppress TH levels, however did not significantly change plasma THs compared to control fish during the experiment.

Condition factor (K)

The condition factor (Fulton, 1902) of sea bream at the start and end of the experiment was determined. This metric, which gives a general idea of fish physiological well-being was unaffected by the short term treatment with THs as no significant differences were found between treatment groups and the control (fig. 4A). It seems therefore, that hormonal treatment and also the inevitable stress caused by manipulation/injection did not have a marked physiological effect that might lead to misinterpretation of the results.

Total protein

Total plasma protein measurements using the Lowry method did not reveal a significant difference in protein concentration between fish subjected to different hormone treatments (fig. 4B and table 1). This indicates that increased levels of THs do not cause a significant alteration in the concentration of total plasma protein in sea bream.

Plasma TTR concentration

This is the first time that circulating TTR has been quantified in fish. The concentration of plasma TTR in control sea bream was 3.8 µg/ml (table 1). The percentage of total plasma protein (~40 mg/ml) which correspond to TTR in sea bream was 0.009 % (n=3). Treatment of sea bream with T_4, T_3 and MMI caused a significant increase ($p<0.001$ for T_3 and $p<0.005$ for

T_4 and MMI) in circulating TTR levels compared to the control fish which were given coconut oil implants alone (fig. 4C and table 1).

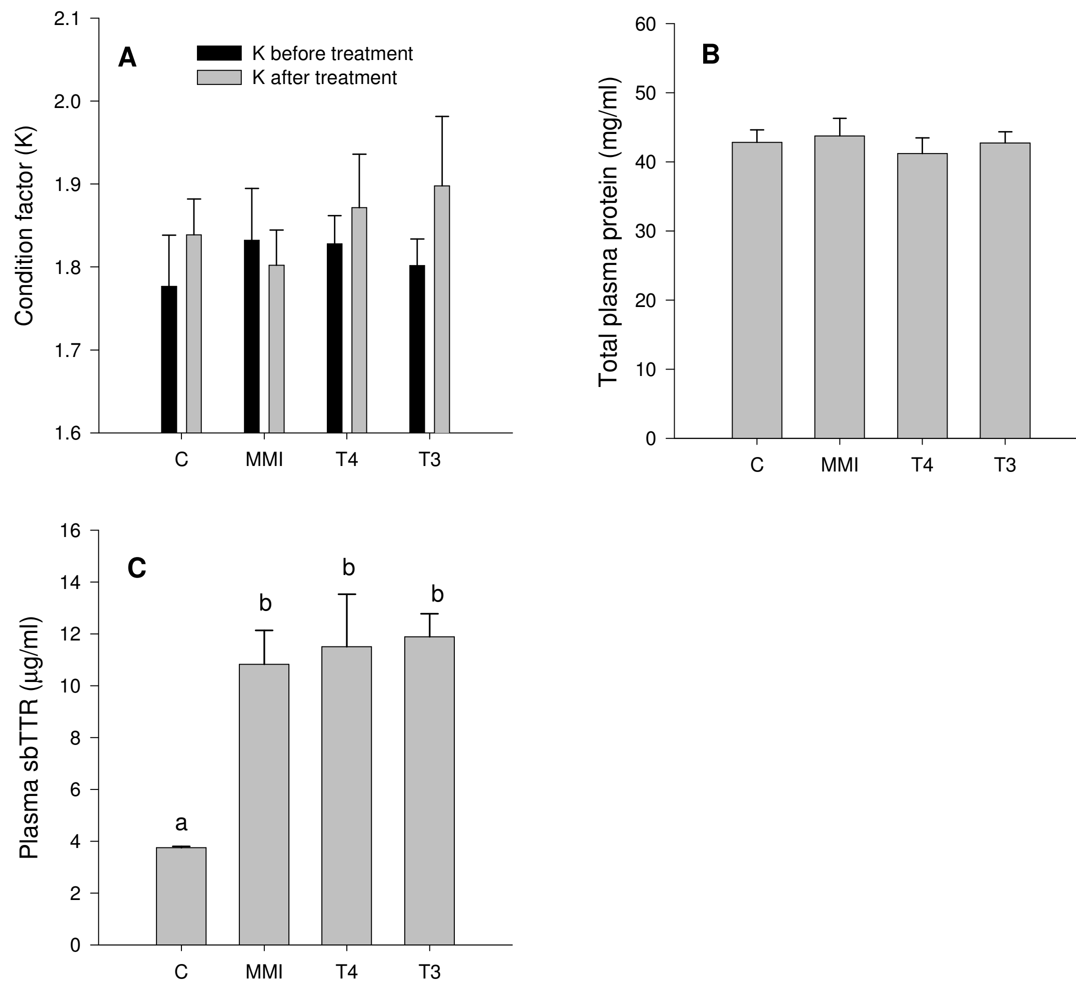


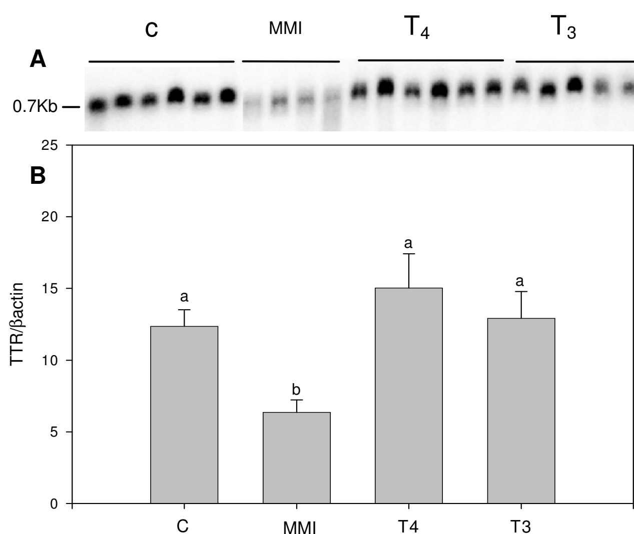
Figure 4. A- Change in condition factor (K= 100 x W x L $^{-3}$) of the fishes before and 10 days after being submitted to the treatments: C – control with coconut oil implant, MMI – methimazol, T_4 – thyroxine, T_3 - triiodothyronine. No significant alteration in K was found between fish receiving hormone/blocker implants and control fish ($p > 0.05$). **B**- Total plasma protein concentration measured in the fishes at the end of the experiment. No changes in protein levels were observed between any of the treatment groups or the control fish ($p > 0.05$). **C**- Plasma transthyretin (TTR) levels for each group (same as previous) by the end of the experiment. Significant differences were found between the treatment groups, T_4 ($p<0.005$), T_3 ($p<0.001$), methimazole ($p<0.005$), and the control fish. No significant differences ($p>0.05$) were found in plasma TTR concentrations in T_4, T_3 and MMI treated fish. Error bars refer to standard error (SE).

Table 1- Concentration of total plasma protein, circulating THs and TTR in each of the experimental groups. Values are given as the mean ± SE. Number of samples analysed are indicated in brackets

Treatment	T_4 (ng/ml)	T_3 (ng/ml)	Total (mg/ml)	TTR (µg/ml)
T_4	1324.1±150.2 (6)	85.9±17.4 (6)	41.2±2.3 (6)	11.5±2.0 (3)
T_3	145.2±18.3 (6)	901.0±196.2 (5)	42.7±1.6 (6)	11.9±0.8 (6)
MMI	56.0±12.8 (5)	62.7±12.7 (5)	43.7±2.6 (5)	10.8±1.3 (3)
Control	36.3 ± 11.9 (6)	35.4±5.0 (6)	42.8±1.8 (6)	3.8±0.05(3)

Hepatic TTR mRNA transcription

The results of sea bream TTR hepatic mRNA exposed to [α-^{32}P]dCTP labelled TTR or β-actin autoradiography (Fig.5 A) and relative quantification of sea bream TTR hepatic expression carried out after radiometric analysis are shown in fig. 5 A and B, respectively. T_4 and T_3 treatment did not change hepatic TTR expression in relation to control fish. However, there was a statistically significant ($p<0.05$) reduction in TTR expression in fish injected with MMI compared to all other groups.



Fgure 5 – **A**- Autoradiographic pattern of the Northern blot of sea bream TTR hepatic mRNA from the different experimental groups exposed to [α-^{32}P]dCTP labelled TTR. **B**- Comparison of sea bream TTR hepatic expression (TTR/β-actin ratio) between different treatments: MMI (n=5), T_4 (n=5), T_3 (n=6) and control (n=6). All data was obtained by radiometric analysis. Results are expressed as mean ± SEM and were analysed by One-way ANOVA followed by Tukey test, using SigmaStat software. Statistically significant differences were observed between MMI and all other groups ($p < 0.05$).

Discussion

TTR is known to be involved in TH transport and metabolic processes related to the thyroid axis in fish (Kawakami et al., 2006; Power et al., 2000b; Santos and Power, 1999; Yamauchi et al., 1999). However, studies concerning the direct action of THs on TTR production in fish are inexistent and the present study focused on this issue. One barrier to studying the influence of different physiological factors on TTR in fish is the lack of tools with which to measure it. For this reason in the present study a recombinant TTR protein from a teleost fish, sea bream, was produced, purified and used to raise specific antisera against the protein. A sensitive and specific ELISA was developed for sea bream TTR and used to measure circulating levels for the first time in a teleost fish. A concentration of 3.8 μg/ml of plasma TTR in juvenile sea bream (table 1, control group) was found which is approximately 100 times lower than the reported concentrations for human plasma TTR (0.2-0.3 mg/ml) determined by different immunological methods (Ingenbleek and De Visscher, 1979; Ritchie et al., 1999; Sachs and Bernstein, 1986) including ELISA (Purkey et al., 2001) which is the method of choice for clinical analysis. The low percentage of total plasma protein which corresponds to TTR in sea bream (~0.009 %) was unsurprising as albumin is the principal protein in blood and is far more abundant than other plasma proteins in all the species studied so far.

The concentration of plasma THs in control fish detected by specific radioimmunoassay was coherent with values previously reported for sea bream using a similar assay (Power et al., 2000b). Very similar concentrations of T_3 and T_4 (35.4 ± 5.0 and 36.3 ± 11.9 ng/ml, respectively) were found in sea bream plasma, which contrasts with the situation in humans where plasma T_4 (50-120 ng/ml) is markedly higher than T_3 (0.95-1.90 ng/ml). The concentration of TTR in humans is 1000 times higher than the concentration of plasma THs so that the ratio of TH:TTR (approximately 85 ng/ml:250 μg/ml) in humans is much lower

than the ratio of TH:TTR (approximately 35.8 ng/ml:3.8 μg/ml:) in fish. The biological significance of this difference will require further study but may be related to a number of factors such as; differences in the abundance and affinity of other plasma THBP in these species; the TH:TTR affinity (Chang et al., 1999; Yamauchi et al., 1999) and also the possibility that TTR evolved to acquire different and wider functions in mammals, such as RBP transport (Kanai et al., 1968; Peterson, 1971; van Jaarsveld et al., 1973). For example, the affinity of TTR is greatest for T_4 in mammals, while in masu salmon it has greatest affinity for T_3 (Yamauchi et al., 1999) and in sea bream it has similar affinity for both T_3 and T_4 (Morgado et al., 2006). Only 1 % of circulating human TTR is involved in TH transport in humans (Robbins J., 1986), in fish the proportion of THs which circulate bound to TTR is unknown, as is its relative importance as a THBP and it also remains to be established if piscine TTR also transports RBP (Folli et al., 2003).

TH implants were highly effective in increasing T_4 and T_3 plasma levels after 10 days of treatment. The administration of T_4 did not cause a significant change in the concentration of plasma T_3 in sea bream, although in rainbow trout (Fok and Eales, 1984) and rabbitfish (Ayson and Lam, 1993) it does, possibly as a consequence of increased conversion of T_4 by 5´-monodeiodination. In fact in teleosts this is the main process responsible for the production of T_3 (Eales and Deborah, 1989; Eales and Shostak, 1985) and this enzyme activity has been found in many species of fish (Leatherland, 1990). In sea bream with increased levels of either T_3 or T_4 there was a significant, approximately 3 fold, increase in circulating levels of TTR, although hepatic transcription was unchanged. This result shows that in this species an increase in plasma THs (several days) is enough to alter circulating levels of TTR probably by changing the rate of secretion and indicates a potential regulatory mechanism. Previous findings in vertebrates indicate a negative thyroid control over TTR production and physiological alterations caused by food deprivation (Eales, 1988) or inflammation (Dickson

et al., 1982; Ritchie et al., 1999) which cause a reduction in THs are also accompanied by a reduction in plasma TTR. The present study supports previous observations in vertebrates that the thyroid exerts control over TTR, but in contrast to mammals, in fish the regulation is positive.

The results of the present study appear to support the theory of "free TH cellular uptake" (Ekins et al., 1982; Mendel, 1989) which proposes that free circulating hormone and not protein-bound hormone is available for entry into cells. THs are lipophilic and can rapidly diffuse into tissues and for this reason binding to transport proteins plays an important role in homeostasis of plasma THs. As TTR is a TH transporter in fish (Kawakami et al., 2006; Santos and Power, 1999; Yamauchi et al., 1999), it regulates free plasma levels of THs and in this way is involved in regulation of the thyroid axis. The increased concentrations of circulating TTR observed in the present study should help to restrain the acute increase in free THs levels induced by treatments.

Both T_3 and T_4 treatments caused a similar increase in circulating TTR in sea bream presumably to maintain a similar bound/free hormone ratio. The results of the present study and those from previous studies in fish, in which measurable T_3 circulates in plasma (Eales and Brown, 1993; Power et al., 2000b) raises the intriguing possibility that in fish the more effective hormone transport system found in mammals in which T_4 acts as a precursor of the active hormone T_3 has not fully developed. The differences in evolution of the thyroid axis between fish and tetrapods may be more fully elucidated by a better understanding of the function of deiodinase processing in TH target tissues and the rest of the thyroid axis in fish.

None of the treatments caused a significant change in plasma total protein compared to the control fish despite the increased levels of TTR. This is not surprising as in control fish TTR corresponds to only ~0.009% of total plasma protein and the 3-fold increase in treated fish would not be detectable with the Lowry assay. No broad fluctuations in any other plasma

proteins levels were found though it has previously been suggested in mammals that the THBP system might act through differential control of these proteins and that any imbalance of the system would be compensated by a change in another element (Schreiber, 2002a). In fish albumin (Babin, 1992) and TTR (Santos and Power, 1999) are the only THBPs identified so far as thyroxin binding globulin (TBG) has yet to be identified. Far more studies will be required of the THBPs in fish in order to characterise how they interact and are regulated and their relative importance in homeostasis of the thyroid axis in fish.

Unexpectedly, MMI a known thyroid axis disrupter in mammals did not appear to block the production of THs in sea bream. In general, inhibitory effects on larval teleost development of antithyroid agents such as, propylthiouracil, thiouracil and thiourea which in common with MMI are thionamides have been reported (Dales and Hoar, 1954; Honma and Murakawa, 1957; Woodhead, 1966). MMI has been found to interfere with zebrafish development (Osama and Klaus, 2003). In fish, post larvae goitrogen interference with TH levels has not been found (Liu and Chan, 2002) and may indicate that thyroid axis regulation in adult fish permit them to overcome the effect of MMI. The significant increase in plasma TTR levels in sea bream coupled to a reduction in liver TTR gene expression caused by MMI treatments may be caused by the toxicity of this goitrogen (Crane et al., 2006). It will be of interest to establish if in common with mammals, TTR in fish acts as an acute response protein (Ingenbleek and Bernstein, 1999).

In conclusion, the concentration of TTR in fish plasma is reported for the first time using a specific ELISA. It would appear that THs regulate hepatic TTR secretion but not gene transcription, although it was not possible to establish in the present study if this was a direct or indirect effect. Moreover, it remains to be established at what level this regulation occurs and the pathways therein involved. That THs failed to influence the total concentration of plasma proteins of which albumin, another THBP, is a significant proportion suggests

differential regulation of their production may occur. That MMI failed to act as an antithyroid agent in sea bream was unexpected and clearly merits further investigation.

Acknowledgements

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Disruption of thyroid hormone binding to sea bream recombinant transthyretin by ioxynil and brominated flame retardants

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2007. **69** (1): p. 155-163

Abstract

A number of chemicals released into the environment share structural similarity to the thyroid hormones (THs), thyroxine (T_4) and triiodothyronine (T_3) and it is thought that they may interfere with the thyroid axis and behave as endocrine disruptors (EDs). One of the ways by which such environmental contaminants may disrupt the TH axis is by binding to TH transporter proteins. Transthyretin (TTR) is one of the thyroid hormone binding proteins responsible for TH transport in the blood. TTR forms a stable tetramer that binds both T_4 and T_3 and in fish it is principally synthesized in the liver but is also produced by the brain and intestine. In the present study, we investigate the ability of some chemicals arising from pharmaceutical, industrial or agricultural production and classified as EDs, to compete with $[I^{125}]$-T_3 for sea bream recombinant TTR (sbrTTR). Ioxinyl, a common herbicide and several polybrominated diphenyl ethers (BDEs) were strong inhibitors of $[I^{125}]$-T_3 binding to TTR and some showed even greater affinity than the natural ligand T_3. The TTR competitive binding assay developed offers a quick and effective tool for preliminary risk assessment of chemicals which may disrupt the thyroid axis in teleost fish inhabiting vulnerable aquatic environments.

Introduction

Environmental contamination by endocrine disruptors is presently a major issue of concern. Such synthetic chemicals can mimic or block hormones interfering with the endocrine system and eventually compromising crucial biological processes. The increasing contamination of the biosphere with chemicals with potential endocrine disrupting effects can become a serious threat to human and wildlife populations.

Thyroid hormones (THs) are known to play a crucial role in many metabolic processes and are essential for normal growth, differentiation and development of vertebrates (Hadley, 1996). Their importance in early life stages is well established in mammals and in amphibians THs are crucial for metamorphosis (Shi et al., 1996). In fish THs are implicated in reproduction and appear to be important in the regulation of development. High concentrations are present in fish eggs and increased levels are reported during metamorphosis or during the larval-juvenile transition (for review see Leatherland, 1994; Power et al., 2001; Yamano, 2005). In light of this evidence, disruption of the thyroid axis may seriously compromise normal development, differentiation, growth or reproduction in many vertebrates (Boas et al., 2006; Brown et al., 2004).

The thyroid hormones 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). 3, 5, 3'-L-triiodothyronine (T_3) is the most active hormone and binds with high affinity to nuclear receptors (TRs), while L-thyroxine (T_4), which is the precursor of T_3 binds with low affinity and has few direct actions (Darras et al., 1998; Hadley, 1996). Almost all THs circulating in the plasma are bound to transporter proteins (Larsson et al., 1985) and only free hormones enter cells to elicit a response (Ekins et al., 1982; Mendel, 1989). The balance of free to bound THs in the plasma depends on plasma proteins, although the importance of this interaction is not entirely understood.

In vertebrates, thyroid hormone-binding proteins (THBPs) include transthyretin (TTR), thyroxine-binding globulin (TBG) and albumin (ALB). Transthyretin in its tetrameric form transports THs and is the major plasma TH carrier in rodents and the main THBP in cerebrospinal fluid of both rodents and humans (Schreiber and Richardson, 1997). In small eutherians and lower vertebrates both albumin and transthyretin seem to be important for TH transport (Power et al., 2000a). However, in tadpoles and fish, TTR is proposed to be the major TH carrier protein (Yamauchi et al., 1993; Yamauchi et al., 1999) and is reported to have higher affinity for T_3 than T_4 in birds, reptiles and amphibians (Chang et al., 1999; Yamauchi et al., 1993; Yamauchi et al., 2000). The TH affinity of TTR in teleost fish is less clear cut and in some species it is reported to have higher affinity for T_3 (Yamauchi et al., 1999), although sbrTTR binds T_3 and T_4 with similar affinities (Morgado et al., 2006).

A number of chemicals released into the environment share structural similarity with THs (fig.1) and may behave as endocrine disrupting chemicals (EDCs) if they substitute THs and disrupt the thyroid axis. Many of these chemicals, usually benzenic halogenated compounds, arising from either the industrial, medical or agricultural sector have been reported to interact strongly with plasma THBPs, especially TTR in vertebrates (Brouwer et al., 1999; Cheek et al., 1999; Ingbar, 1958; Ishihara et al., 2003a; Ishihara et al., 2003b; Lans et al., 1993; Rickenbacher et al., 1986; Yamauchi et al., 2000). Other compounds structurally even closer to THs are the brominated flame retardants (BFRs), extensively used in electronic and plastic materials. These organohalogenic chemicals are produced as commercial mixtures that include a wide variety of bromine-containing derivatives, like the polybrominated diphenyl ethers (PBDE) or tetrabromobisphenol A (TBBPA), and many of them are found as persistent pollutants in the environment with bioaccumulating potencies.

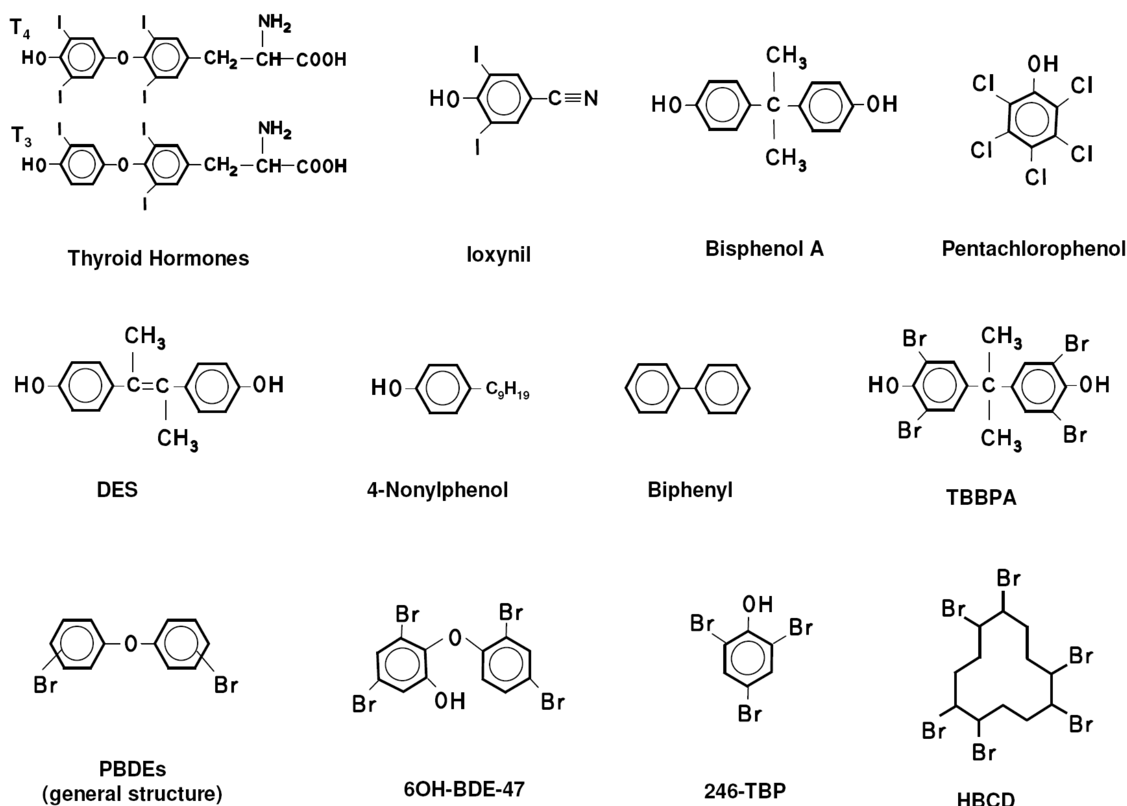

Figure 1. Structure of thyroid hormones and several chemicals (potential endocrine disruptors) tested for their capacity to compete with sbrTTR-[^{125}I]-T_3 binding in the *in vitro* assay.

In the past few years BFRs have been associated with endocrine disruption (Darnerud et al., 2001; Hamers et al., 2006) and many of them were found to mimic THs (Kitamura et al., 2002; Zhou et al., 2002) and bind human TTR *in vitro* (Hamers et al., 2006; Meerts et al., 2000).

The binding and transport of exogenous compounds by THBPs might be expected to affect TH balance as they may be preferentially taken up by cells leading to thyroid axis disruption. The aquatic environment and organisms such as fish are constantly threatened by pollution resulting from human activity and a previous study has shown that EDCs can bind to TTR in masu salmon (Ishihara et al., 2003b). The large number of chemicals released into the aquatic environment (from industrial residues, land run-offs and acid rains) and the desire of society

to minimize the use of animals for testing highlights the need for quick robust and cheap predictive *in vitro* assays.

In the present study a competitive binding assay was developed and the effect of putative EDCs of agricultural, medical and industrial origin on $[I^{125}]$-T_3 binding to sbrTTR was assessed. Teleost fish are frequently used as sentinel organisms for aquatic ecosystem contamination and the present *in vitro* assay with teleost TTR should be a more adequate risk assessment tool than equivalent assays with mammalian TTR.

Materials and Methods

Reagents

Labeled $[^{125}I]$-T_3 (114 MBq μg^{-1}) was purchased from Amersham Biosciences (UK). Unlabeled T_3, T_4, ioxynil and 4-nonylphenol were obtained from Sigma-Aldrich (Madrid, Spain). Pentachlorophenol, Diethystilbestrol, Biphenyl and Bisphenol A were acquired from Cymit Química, S.L. (Barcelona, Spain). A suite of BFRs were purchased or synthesised (table 1). Hormones and all the compounds tested as EDCs were dissolved in dimethylsulfoxide (DMSO) to a concentration of 2 mM or 2.5 nM and then diluted in the assay buffer; the concentration of DMSO in assays was always less than 1% (v/v). Control experiments were carried out with DMSO alone in the same concentration range as used for assays with EDCs, in order to assess if it influenced $[^{125}I]$-T_3 binding to sbrTTR. None of the concentrations of DMSO tested influenced binding.

Table 1. Source of the brominated flame retardants (BFRs) tested for sbrTTR binding ability

BFRs	Acquired from
BDE-28, BDE-47, BDE-49, BDE-99, BDE-100, BDE-153, BDE-155, BDE-169, BDE-181, BDE-183, BDE-190, BDE-206, BDE-209, 6-OHBDE-47	Stockholm University (Dr. Göran Marsh and Dr. Ake Bergman)
BDE-19, BDE-38, BDE-39, BDE 127, BDE 185	Accu Standard
Pentamix	Great Lakes Chemical Corporation
TBBPA, 246TBP	Aldrich
HBCD	Dead Sea Bromine group
αHBCD, βHBCD, γHBCD	Cambrige Isotope Laboratories
TBBPADBPE	Broomchemie BV Terneuzen

Production and isolation of sbrTTR

Recombinant protein expression was carried out in *E. coli* strain BL21DE3pLysS transformed with the expression vector construct pET24d(+)/sbTTRWT by IPTG (0.2 mM) induction as described in chapter 2. After cell lysis the protein extract was analysis on SDS-PAGE and further purified, concentrated, dialyzed and submitted to western blot with colorimetric detection with specific anti-sbrTTR antisera. All these procedures are given in detail in the general methods section (chapter 2).

Analysis of [^{125}I]-T_3 binding by sbrTTR using native PAGE

To establish if the purified sbrTTR was able to bind ligand it was incubated on ice for 2 h with [^{125}I]-T_3 (0.01 μCi); a parallel reactions with [^{125}I]-T_4 (0.01 μCi) and a human plasma sample (1.5 μl) which contain albumin (67 kDa) that binds T_4 was also conducted. Subsequently, binding reactions were subject to electrophoresis on 12.5 % native glycine-acetate gels run at a constant current (60 mA) in glycine-acetate 1 x running buffer (see

appendix). The gel was then dried under vacuum for one hour at 60-80 ºC (Drygel.jr se540, Hoeffer Scientific Instruments, UK) and exposed for 48 h at -80 ºC to Kodak BioMax MS film in a cassette containing an intensifying screen.

[^{125}I]-T_3 Competitive Binding assay

For competitive binding studies, sbrTTR (50 ng per tube) was incubated in 200µl TCN buffer (see appendix) containing tracer 0.1 nM [^{125}I]-T_3 in the presence of increasing concentrations of unlabeled ligand T_3, or EDCs (0-10 µM) for 2 h on ice. Separation of free and sbrTTR bound [^{125}I]-T_3 was carried out as described in chapter 2- Ligand binding assay section. Assays were done in duplicate and each chemical was tested on at least three different occasions. Results were analysed using One-way analysis of variance (ANOVA). If statistically significant differences were detected between treatments, a Tukey (HSD) multiple comparison test was applied. All the statistical analysis was performed using Sigma Stat software version 3 (SPSS). Differences were considered statistically significant at $p<0.05$.

Results

Production of pure sbrTTR and [^{125}I]-T_3 binding in native PAGE

sbrTTR for use in binding assays was obtained after expression optimization under 0.2 mM IPTG induction of recombinant *E. coli* BL21DE3pLysS at 30 ºC (fig. 2A-a). The sbrTTR was purified from bacterial extracts by continuous-elution native electrophoresis and a single band of the expected size for the monomer (14 kDa) was identified analytical SDS-PAGE 15% (reducing conditions); no additional proteins were detected even though the extremely sensitive silver staining method was used (fig.2 A-b). Western blot of the purified protein using specific sbrTTR antisera revealed a single immunoreactive protein of the expected size

(fig. 2A-c). Concentration of total protein in the sbrTTR purified sample (4 ml final volume), determined using the molar extinction coefficient at 280 nm and was found to be 0.25 mg/ml. Nondenaturing PAGE of the binding reactions of human plasma incubated with $[^{125}I]$-T_4 or sbrTTR with $[^{125}I]$-T_3 gave a single intense band respectively after autoradiography and had a different migration pattern from labelled hormone alone (fig. 2B); the $[^{125}I]$-T_3 bound to sbrTTR could be displaced by excess unlabelled hormone (not shown). The recombinant sbrTTR was found to migrate slightly faster than human plasma albumin (67 kDa) with a pattern typically described for tetrameric TTR in most other species (Chang et al., 1999; Kabat et al., 1942); TTR is not visible in the human plasma sample as it is found in blood in much lower concentrations than albumin.

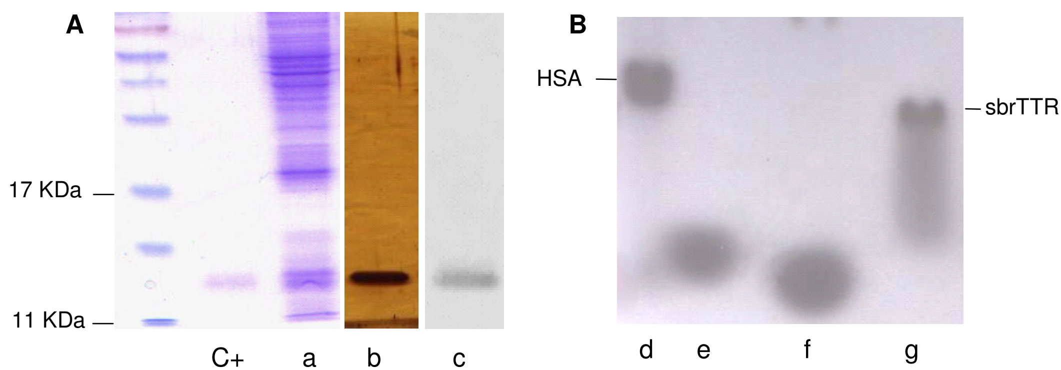

Figure 2. A - Analysis of sbrTTR production and purification. a) SDS-PAGE of protein extracts prepared from recombinant bacteria after induction with IPTG; previously purified sbrTTR sample was used as a marker for comparison (first lane) b) sbrTTR purified and analysed by SDS-PAGE (15%) followed by silver staining, or by c) western blot with specific anti-sbrTTR sera. **B** – PAGE under native conditions of ligand-binding experiments with human plasma and sbrTTR incubated respectively with $[^{125}I]$-T_4 or $[^{125}I]$-T_3. A single intense band of d) human albumin (approx. 67 KDa) + $[^{125}I]$-T_4 and g) sbrTTR + $[^{125}I]$-T_3 were identified after autoradiography. A single band migrating with the "electrophoretic front" was detected for e) $[^{125}I]$-T_3 and f) $[^{125}I]$-T_4.

Effect of EDCs on [125I]-T_3 binding to sbrTTR

Competitive binding experiments with the pure recombinant protein using [125I]-T_3 revealed that binding to sbrTTR was displaced by increasing concentrations of unlabeled T_3 and T_4. Moreover, both T_3 and T_4 displaced [125I]-T_3 from TTR with similar efficiency ($p>0.05$) and had IC_{50} values of 23.8 ± 5.4 nM and 20.9 ± 3.7 nM, respectively (fig. 3 and tables 2 and 3). In competitive binding assays with putative EDCs, [125I]-T_3 binding to sbrTTR was displaced by increasing concentrations of a number of the tested chemicals.

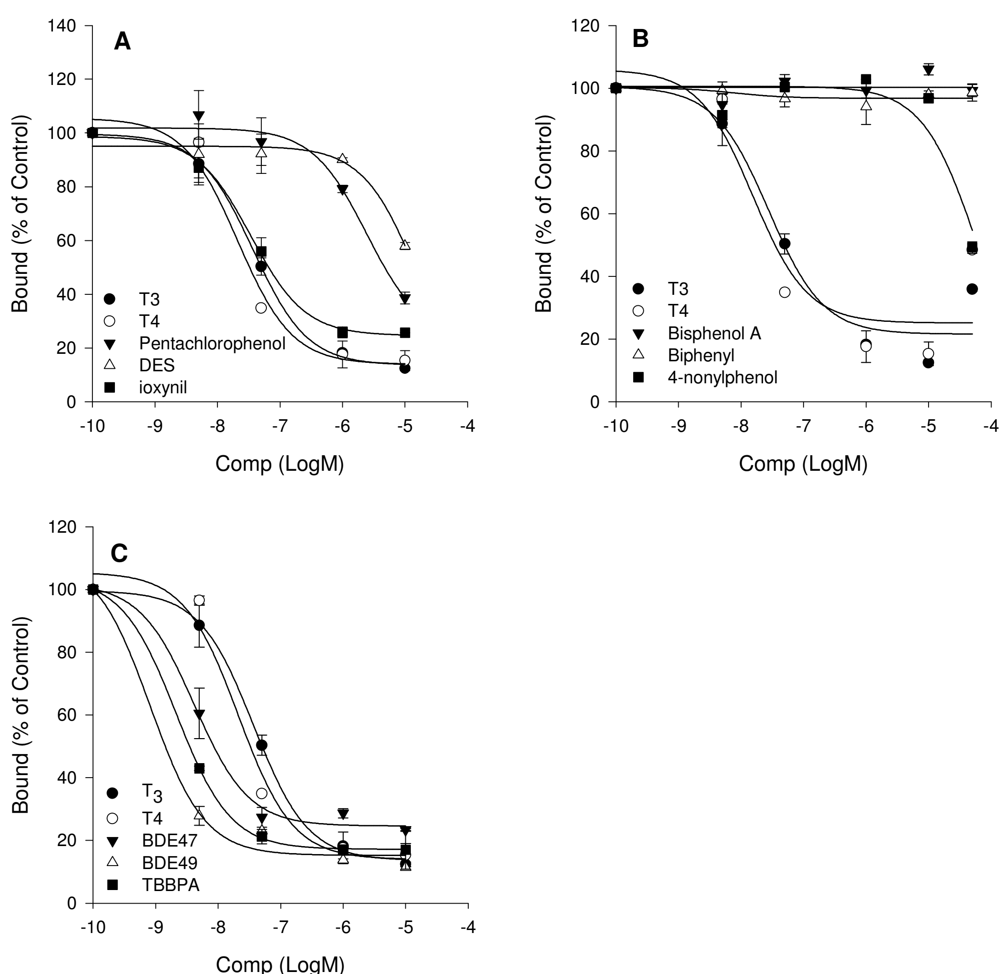

Figure 3. Competitive inhibition curves of [125I]-T_3 binding to sbrTTR by EDCs. SbrTTR was incubated for 2 h on ice with 0.1 nM [125I]-T_3 in the presence of the following compounds: **A-** (●) T_3, (○) T_4, (▼) pentachlorophenol, (Δ) DES, (■) ioxynil. **B-** (●) T_3, (○) T_4, (▼) Bisphenol A, (Δ) Biphenyl, (■) 4-nonylphenol. **C-** (●) T_3, (○) T_4, (▼) BDE-47, (Δ) BDE-49 and (■) TBBPA. Total binding is shown as a percentage of the control and each value is the mean of two duplicates.

The brominated flame retardants, TBBPA and BDE-49, 47 and 99, were the most potent inhibitors of [^{125}I]-T_3 binding to sbrTTR with IC_{50} values significantly lower ($p<0.05$ for BDE-47 and 99; $p<0.001$ for TBBPA and BDE-49) than the natural ligand T_3 and T_4 (fig. 3C and table 3). Ioxynil, a common herbicide (fig. 3B and table 2), and a panel of several other BFRs, mostly BDEs but also pentamix and 246TBP (table 3) were also strong competitors for [^{125}I]-T_3 binding to sbrTTR and their IC_{50} values did not differ significantly ($p>0.05$) from that of THs, suggesting they have similar affinity for the protein (tables 2 and 3). 6OH-BDE-47 was a moderate competitor that could displace [^{125}I]-T_3 binding to sbrTTR although with a significantly higher ($p<0.001$) IC_{50} value (700.6 ± 40.4 nM) than THs (table 3). Pentachlorophenol, Diethylstilbestrol (DES) and 4- Nonylphenol were poor competitors showing much higher IC_{50} values (table 2) than T_3 and T_4 ($p<0.001$). Bisphenol A and Biphenyl and many of the BFRs tested did not show binding to sbrTTR as assessed by their inability to displace [^{125}I]-T_3 binding to the protein. The rank order of hormone and chemicals binding to sbrTTR is described in tables 2 and 3.

Table 2. sbrTTR binding ability to THs and several putative EDCs. The IC_{50} values are indicated in rank order of binding affinity expressed as mean ± S.E. (n = 4 for THs and ioxynil and n = 3 for the tested chemicals). The IC_{50} of T_3 and T_4 binding to sbrTTR was not significantly different ($p>0.05$).

Chemicals	**IC_{50} (nM)**
T_4	20.9 ± 3.7
T_3	23.8 ± 5.4
Ioxynil	38.9 ± 4.9
Pentachlorophenol *	2480 ± 1023
Diethystilbestrol *	4849 ± 1841
4-Nonylphenol *	17035 ± 6865
Bisphenol A	-
Biphenyl	-

*significantly different from THs binding ($p<0.001$)
- no effect

Table 3. IC_{50} values of THs and Brominated flame retardants (BFRs) tested for T_3-sbrTTR competing capacity expressed as mean ± S.E. (n=3) in rank order of binding affinity. The IC_{50} of T_3 and T_4 binding to sbrTTR was not significantly different (p>0.05).

BFRs	IC_{50} (nM)	BFRs	IC_{50} (nM)
T_4	20.9 ± 3.7	BDE 153 [2,2',4,4',5,5']	-
T_3	23.8 ± 5.4	BDE 127 [3,3',4,5,5']	-
BDE49 [2,2',4,5']**	0.5 ± 0.3	BDE 155 [2,2',4,4',6,6']	-
TBBPA**	2.1 ± 0.3	BDE 169 [3,3',4,4'5,5']	-
BDE 47 [2,2',4,4']*	5.25 ± 0.7	BDE 181 [2,2',3,4,4',5,6]	-
BDE 99 [2,2',4,4',5]*	6.7 ± 2.4	BDE 183 [2,2',3,4,4',5',6]	-
Pentamix	8.3 ± 4.5	BDE 185 [2,2',3,4,5,5',6]	-
BDE 39 [3,4',5]	9.5 ± 2.4	BDE 190 [2,3,3',4,4',5,6]	-
BDE 28 [2,4,4']	14.9 ± 10.8	BDE 206 [2,2',3,3',4,4',5,5',6]	-
246TBP	30.0 ± 13.6	BDE 209 [2,2',3,3',4,4',5,5',6,6']	-
BDE 19 [2,2',6]	43.9 ± 17.8	TBBPADBPE	-
BDE 100 [2,2',4,4',6]	55.5 ± 28.9	HBCD	-
BDE 38 [3,4,5]	65.1 ± 24.3	αHBCD	-
60HBDE47**	700.6 ± 40.4	βHBCD	-
		γHBCD	-

*Significantly different than THs binding with p<0.05
**Significantly different than THs binding with p<0.001
- no effect

Discussion

Many hydroxylated compounds, like PCBs, have been shown to have endocrine disrupting activity on the thyroid axis and TTR binding abilities in mammals, birds, amphibians and fish (Brouwer et al., 1998; Brown et al., 2004; Ishihara et al., 2003a; Ishihara et al., 2003b; Yamauchi et al., 2000). In the present study, different compounds frequently found as environmental pollutants and some of which have previously been reported to bind human TTR *in vitro* were tested for their ability to interfere with $[^{125}I]$-T_3 binding to sbrTTR (table

1). Bisphenol A and 4-Nonylphenol are widely used in the plasticizers industry and have been found as contaminants mainly in aquatic environments. Pentachlorophenol, Biphenyl, ioxynil, common pesticides, and DES a synthetic estrogen are also identified environmental contaminants. BFRs are also recognized as persistent contaminants and a large panel of such chemicals was tested in the present study.

This is the first time that the disruptive potential of the aforementioned compounds has been tested with a piscine TTR and results indicate that the competitive binding assay developed may be a useful indicator of potential thyroid axis disrupting compounds. The use of an *in vitro* TTR binding assay as a screening method for detection of EDCs in environmental waste water was previously suggested using avian and amphibian plasma TTRs (Yamauchi et al., 2003). Also, systematic *in vitro* screening for TTR-binding potency of BFRs was recently performed using human TTR (Hamers et al., 2006). Here we propose that the use of a piscine TTR assay may be more appropriate for determination of TTR-binding potency of BFRs or other EDs in fish, which are frequently used as sentinel species for assessment of environmental quality. Such an idea acquires greater significance when the TH binding characteristics of piscine and other vertebrate TTRs is considered (Yamauchi et al., 1999). Despite the high amino acid conservation of TTR's binding channel between different organisms, in terrestrial vertebrates the protein has a significantly greater affinity for T_4 than for T_3 and in fish no significant difference or a slightly greater affinity for T_3 has been found (Morgado et al., 2006; Yamauchi et al., 1999).

The present results with sbrTTR underline the relevance of a specific piscine TTR assay as a more adequate indicator of hazard for fish exposed to the test chemicals. Moreover, comparison of the disrupting potential of the compounds on TH binding to TTR between different species reveals significant differences. For example, DES and Pentachlorophenol were powerful inhibitors of [^{125}I]-T_3 binding to TTR purified from plasma of chicken (IC_{50}:

DES 0.4 nM, pentachlorophenol 6 nM), bullfrog (IC_{50}: DES 0.3 nM, pentachlorophenol 45 nM) (Ishihara et al., 2003b) and Japanese quail (IC_{50}: DES <0.4 nM, pentachlorophenol 6.3 nM) (Ishihara et al., 2003a). Similar observations were also made with binding of DES to recombinant *Rana* and *Xenopus* TTR which had an IC_{50} of 9 and 400-550 nM respectively (Yamauchi et al., 2000). In contrast, sbrTTR showed very poor affinity for DES and pentachlorophenol (IC_{50} = 4.8±1.8 and 2.5±1.0 µM respectively). Bisphenol A was a poor inhibitor of TTR-TH binding (IC_{50} from 1500 nM to 10 µM) for all the above mentioned species and it failed to bind to sbrTTR. These observations reinforce the notion of species-specific differences in TTR binding affinities for EDCs and it is noticeable that avian (chicken or quail) TTRs are more susceptible to EDCs than bullfrog TTR. The same kind of comparison was also established by Ishihara, et al (2003b) using THBP serum samples extracted from human, chicken (plasma and cerebrospinal fluid), bullfrog and masu salmon and by determining their relative [^{125}I]-T_3 binding inhibition potencies. Bisphenol A, a poor EDC binder for other species was found to be a potent inhibitor of [^{125}I]-T_3 binding to human THBP. TBG is the major THBP in human plasma which has generally greater resistance to EDC binding than avian, amphibian or fish plasma in which TTR is the major THBP. Such observations indicate that when plasma samples are utilised in binding assays the differing affinities encountered may not only be a consequence of TTR affinity but also of the relative abundance and importance of other THBP (Brucker-Davis, 1998).

The affinity of sbrTTR for some compounds like TBBPA, 246-TBP, ioxynil or pentachlorophenol is consistent with a proposed structure-activity relationship, in which an increase in interaction with TTR is observed for chemicals with hydroxylations in a *para* position, with one but preferably two adjacent halogens and this affinity increases with the number of halogen substitutions, especially bromine over chlorine (see fig.1). The latter type of structure seems to be required for human TTR binding to hydroxylated PCBs (Lans et al.,

1993) and brominated bisphenols (Meerts et al., 2000). This structure-activity relationship could also explain the poor binding affinity of sbrTTR for 4-Nonylphenol, Bisphenol A, Biphenyl or DES (fig.1).

We also report extremely strong affinities between sbrTTR and several BDEs, ranging from BDE-19 to BDE-100, which include tri, tetra and pentabromodiphenylethers, especially BDE-49, 47 and BDE-99 with four and five bromine substitutions respectively. Such binding ability, however, seems to be lost for BDEs with higher degrees of bromine substitution (table 3).

The affinity of BDEs for sbrTTR is quite different from human plasma TTR where very low binding affinities for these compounds (IC_{50} values over 7000 nM) were found (Hamers et al., 2006). In fact, BDEs have a favourable bromine substitution but lack the characteristic *para* hydroxylation commonly found in strong TTR binders. The results of the present work are however in agreement with previous studies using bromophenols and non-hydroxylated compounds which showed that strong binding to TTR can occur even in the absence of OH group interactions (Ghosh et al., 2000; Rickenbacher et al., 1986). Crystallographic studies revealed that the above mentioned bromophenol compounds bind to human TTR in an unusual “reversed” mode where the hydroxyl group doesn’t seem to play an important role (Ghosh, Meerts et al. 2000) which may explain the unexpected affinities of the none-hydroxylated BDEs in ligand-binding studies.

The reason for the different binding affinities of BFRs to human and piscine TTR remains to be established but it seems likely that differences in the physicochemical and structural properties are important. The crystal structure of sbrTTR is available (Eneqvist et al., 2004; Folli et al., 2003) and its structure is well conserved with that established for human, rat or chicken. However, the same study reveals that despite amino acid conservation (only one substitution) the shape of the TTR hormone binding channel is different when compared to

human TTR (Eneqvist et al., 2004). Also the surface potential of sea bream and chicken TTR seems to be more negative than the human or rat proteins (Power et al., 2000a). These facts could explain discrepancies in binding affinities between such species. Moreover, it is well known that the N-terminal amino acid sequence is shorter and more hydrophilic in eutherians, and longer and more hydrophobic in birds, reptiles, amphibians and fish (with three additional amino acids). The significance of this difference has been widely discussed and it has been proposed to strongly influence the TH binding properties of TTR (Morgado et al., 2006; Power et al., 2000a; Prapunpoj et al., 2002). In light of the previous findings and since chemical and structural aspects of TTR-TH binding are not completely clarified, conclusions that directly relate the chemical structure of sbrTTR to binding affinity are still difficult to draw. Although in general diphenyl compounds with halogen substitutions, which most resemble the structure of THs, seem to have a greater affinity for this protein.

In vitro binding assays provide a relatively quick method, which does not imply the use of animals, to screen for potential ED effects of chemicals. However, such assays have limitations; the poor binding affinity for TTR found for some chemicals does not exclude their possible disrupting activity in a direct or indirect way *in vivo*. For instance, 4-Nonylphenol, ubiquitous in aquatic environments and reported to alter T_4 levels and inhibit growth and development in Atlantic salmon (Fairchild et al., 1999) had a poor *in vitro* TTR binding affinity in the present study. The latter may indicate that many more mechanisms other than TTR-binding can lead to thyroid-axis disruption. For example, EDCs may act by binding to other THBP and bioaccumulation and metabolism may also modulate their effects. It has previously been observed that some metabolites of EDCs become even stronger TTR binders than the original compounds (Brouwer and van den Berg, 1986). A similar situation may occur in the environment if EDCs interact with other natural or synthetic chemicals and generate by-products with a stronger endocrine disrupting activity. For example, Yamauchi *et*

al (2003), reported that although Bisphenol A and Nonylphenol show poor binding affinity for chicken and bullfrog TTRs, their respective chlorinated derivatives can be powerful binding inhibitors with affinity increasing with their degree of chlorination (Yamauchi et al., 2003). This means that a poor effect detected in TTR binding assays *in vitro* does not exclude potential harmful effects of compounds *in vivo*.

Many questions are still unanswered concerning the biological significance of EDCs interfering with the thyroid axis. *In vitro* assays or short term exposure may give a measure of hazard but do not reflect the *in vivo* situation. Moreover, data about the concentration of EDCs in aquatic environments is scanty and generally relates to wastewater treatment plant discharges and industrial residues. However, due to the high production volumes (mid-1990s was 600000 metric tonnes) and use of PBDEs (de Boer et al., 2003; Manchester-Neesvig et al., 2001; North, 2004; Oberg et al., 2002) reports of their concentration in river water samples, marine biota and biosolids exist (Akutsu et al., 2001; Anderson and MacRae, 2006; Ikonomou et al., 2002; Lacorte et al., 2003; Manchester-Neesvig et al., 2001).The most commonly detected contaminants are penta-BDE congeners, components of one of the major commercial mixtures Bromkal 70-5DE which include BDE-47, 99 and 100, strong sbrTTR binders in the present study. For example, effluent concentrations of up to 10.5 and 11.2 ng/l have been reported for BDE-47 and 99 respectively in the USA (North, 2004) and in Penobscot River (Main, USA) values of up to 50.0 ng/l were measured (Anderson and MacRae, 2006). Moreover, as BDEs are highly lipophylic they readily bioaccumulate and BDE-47 and 99 concentrations of 10000 ng/g lipid, and BDE-100 at 2000-3000 ng/g lipid have been measured in small mouth bass in Penobscot river. Bioaccumulation of a range of other BDE congeners have been reported in a number of fish species in North America and northern Europe where concentrations tend to be lower (for review see Anderson and MacRae, 2006). The levels of environmental contamination and bioaccumulation of BDEs

suggest that in fish these chemicals may reach values at which they can efficiently out-compete TH binding to TTR and disrupt this aspect of the TH axis.

Further experiments are required to clarify the biological consequences of EDC binding to TTR. Nevertheless, plasma proteins, and particularly TTR because of its role in TH transport and delivery are very likely to strongly influence EDC action in vertebrates. Therefore, the use of preliminary methods to detect putative EDCs based on TTR binding seems to be a pertinent choice and of particular interest for aquatic habitats when using a teleost fish TTR.

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Disruption of the thyroid system by diethystilbestrol and ioxynil in the sea bream (*Sparus aurata*)

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Abstract

Chemicals contaminating the environment are thought to cause disruption of the thyroid system in vertebrates. Endocrine disrupting chemicals (EDCs) may act at several levels in the thyroid axis, synthesis, transport and metabolism of thyroid hormones (THs). Several putative EDCs have a strong affinity *in vitro* for Transthyretin (TTR), one of the thyroid hormone binding proteins (THBPs), although how they behave *in vivo* is unknown. *In vitro* analysis reveals that ioxynil and diethylstilbestrol (DES) strongly bind sea bream TTR and in the present study the aim was to establish how they influence the thyroid axis *in vivo*. DES, ioxynil (1mg/kg fish) and propilthyouracil (PTU, 1 and 10 mg/kg) were administered in the diet to sea bream juveniles (n=14/treatment) for 21 days. Ioxynil slightly increased ($p<0.05$) total protein plasma levels and fish were euthyroid ($p>0.05$) and quantitative PCR analysis revealed that TSH expression was down-regulated in the brain ($p<0.001$) and pituitary ($p<0.005$). Deiodinases DII and DIII expression was strongly down-regulated in the brain ($p<0.001$) but not in the pituitary or the liver (only determined for DII). PTU lowest dose and DES treatment down-regulated TRβ expression in the pituitary. TTR plasma levels remain to be quantified but hepatic expression was up-regulated by PTU lowest dose ($p<0.05$) and unaltered by the other treatments. The histology of the thyroid in PTU (1mg/kg fish) group was indicative of low TH production. It appears that the *in vitro* TTR-binders ioxynil and DES strongly influence peripheral deiodination although the thyroid axis appears to maintain TH homeostasis in the fish. Further analysis of thyroid histology and TTR plasma levels are required to better understand the action of ioxynil and DES.

Introduction

Disruption of the endocrine system by xenobiotic compounds is consistently reported in humans and wild life species and is a matter of concern worldwide. In particular, disruption of the thyroid system, which has had relatively little attention, is now increasingly reported in vertebrates (for review see Boas et al., 2006; Brown et al., 2004; Colborn, 2002). Since thyroid hormones (THs) are crucial for many biological processes (Hadley, 1996) thyroid disruption can seriously compromise normal development of exposed species. In fish THs are thought to be very important in reproduction and regulation of development (Power et al., 2001; Yamano, 2005). A great variety of natural or synthetic chemicals from different classes or sources (e.g. all sorts of pesticides, industrial by-products or pharmaceutical drugs) are thought to exert an acute affect at different levels of the thyroid cascade. It is consensual that endocrine disrupting chemicals (EDCs) act by interfering with TH synthesis, cellular uptake or metabolism, at the level of TH receptors (TRs) and also with TH transport by binding to thyroid hormone binding proteins (THBP) (Boas et al., 2006; DeVito et al., 1999). The TH transport system in particular may be quite susceptible to EDCs as many chemicals are structurally related to THs and may bind THBPs and disturb homeostasis of TH levels or even cellular uptake (Divino and Schussler, 1990). In fact many *in vitro* studies report that TTR, one of the THBPs in vertebrates, has strong affinity for a great number of putative EDCs in human, birds and amphibians (Cheek et al., 1999; Hamers et al., 2006; Ishihara et al., 2003a; Ishihara et al., 2003b; Kudo and Yamauchi, 2005; Meerts et al., 2000; Yamauchi et al., 2003). In particular in fish, where TTR is the major THBP its binding to EDCs could have greater consequences to normal thyroid status. In the present thesis (chapter 5) an *in vitro* sea bream recombinant TTR (sbrTTR) binding assay was developed and a number of chemicals shown to bind the protein more strongly than THs. However, the possible *in vivo* binding of EDCs and the consequences of exposure to such compounds in fish are completely unexplored. In

fact, although many environmental chemicals have been shown to influence the thyroid cascade in more than 40 teleost species (Brown et al., 2004) *in vivo*, mechanisms of thyroid disruption are unclear and have never been explored from the TH transport endpoint perspective.

Two compounds, diethylstilbestrol (DES, a synthetic nonsteroidal estrogen) and ioxynil (a hydroxybenzonitrile herbicide) which bind strongly to sbrTTR *in vitro* (chapter 5) were tested *in vivo*. Ioxynil in particular was found to bind sbrTTR with very high affinity. Previous studies by Ishihara et al. (2003a; 2003b) also report DES and ioxynil amongst the strongest binders *in vitro* to serum protein extracts from humans, chicken, bullfrog and masu salmon. Therefore, in the present study the effect of exposure to two putative EDCs, DES and ioxynil on sea bream thyroid status was assessed. In parallel, the consequence of exposure to propilthyouracil (PTU) a known anti-thyroid drug were also analysed as a positive control of TH inhibition. A multi-parameter assessment of the thyroid axis was used to identify at what level disruption occurs. For this reason total plasma protein, circulating TH concentrations and transcript abundance of TTR, TSH, deiodinases and TRβ were carried out in different tissue.

The current study should help establish the effect of EDCs on fish thyroid status and also provide useful data on the potential toxicity of the tested compounds. This is important information as fish are frequently used as sentinels for pollution in aquatic ecosystems and the physiological importance of THs means that disruption of this axis may threaten their survival.

Methods

Animals and experiment conditions

Juvenile sea bream were obtained from TIMAR (Tavira, Portugal) and maintained in 1000 l open sea water circuits at the experimental station Ramalhete Marine Station (Faro, Portugal) at normal ambient temperature and salinity for the Algarve, Portugal in September. Fish of approximately similar sizes were chosen for the experiments and transferred to glass tanks of 65 L at a density of 14 or 12 fish per tank. All experimental tanks were in closed circuit, each with its own water pump and a second submersible aeration/filtration device (Duetto, Aquarium Systems) to improve oxygen circulation and water quality. A 12 h light/dark photoperiod was maintained with daybreak set at 07.00h. Water temperature was 20-21 ºC during the experiment and salinity 36 parts per thousand. Fish where allowed to adapt to experimental conditions for 2 weeks prior to experiments and were fed twice daily (1.5 % total fish weight per tank per day) with a commercial sea bream diet (Dourasoja size 2 mm). Every 3 days 1/3 of the tank water was substituted and water pumps and filters washed. Before starting the experiment, fish in each group were weighed (g) and length (snout to tail bifurcation) measured and mean body weight ± S.E. and mean body length ± S.E determined. Experimental groups and tanks were labelled with the treatment and dose as follows: PTU1-1 mg/kg fish (n=14; 11.6 ± 0.8 g and 8.7 ± 0.2 cm), PTU10-10 mg/fish kg (n=14; 9.9 ± 0.7 g and 8.3 ± 0.2 cm), DES-1 mg/fish kg (n=14; 11.3 ± 0.8 g and 8.6 ± 0.2 cm), Ioxynil-1 mg/fish kg (n=14; 10.9 ± 0.7 g and 8.6 ± 0.2 cm) and a C-control tank (n=12; 9.1 ± 0.6 g and 8.1 ± 0.2 cm). Treatment with PTU, an antithyroid drug know to inhibit TH synthesis, was used as a positive control. The concentration of each tested dose (1 mg/kg fish or 10 mg/kg fish) corresponds to the cumulative exposure of fish to the test chemicals at the end of the experiment. Experiments were carried out for 21 days and the estimated daily exposure to

chemicals was 0.05 and 0.5 mg/kg fish/day. Maintenance and manipulation of fish was conducted in accordance with the Guidelines of the European Union Council (86/609/EU).

Preparation and administration of the chemicals

Food was chosen as the vehicle for administration of the chemicals and treated food was prepared weekly (to avoid oxidation) and stored at 4 ^{o}C in the dark. Commercial sea bream pellets were sprayed with the appropriate concentration of chemical solution and fed at the desired dose to the fish. To prepare the solution of chemicals they were dissolved in DMSO to give a stock solution of 10 mM which was diluted in ethanol to the appropriate concentration and used to treat the food (maximum concentration <1 % DMSO). The food given to control fish was treated with vehicle alone (ethanol and <1 % DMSO). Food was evenly coated with the chemical solution and left in a fume cupboard until all the ethanol had evaporated. Just before feeding the food was sprayed with cod liver oil to increase its palatability.

Sample collection

After 21 days of treatment fish were anaesthetised in phenoxyethanol (0.1 %; Sigma-Aldrich), weight and length measured and a blood sample removed using heparinized syringes. Blood was centrifuged at 1000 g for 10 min at 4 ºC and the plasma frozen in liquid nitrogen and stored at -20 ºC until analysis. Fish were subsequently killed by decapitation and brain, pituitaries and liver collected, frozen in liquid nitrogen and stored at -80 ºC until utilised for RNA extraction. Tissue from the insertion of the gill arch into the lower mandible was fixed in Bouin-Holland for subsequent thyroid histology.

Sample analysis

Condition factor (K) was determined using the following formula, K= 100 x W x L^{-3} (W = body weight and L = length) and the fish weight and length recorded at the beginning and end

of the experiment. The total protein concentration in plasma was determined using the Lowry Method (Lowry et al., 1951) with bovine serum albumin as the standard. Absorbance was measured at 745 nm. Plasma THs (T_3 and T_4) were determined by radioimmunoassay (RIA) as described in Chapter 2. TTR in plasma was determined by western blot analysis using the ECL system (ECL plus western blotting detection system, Amersham Biosciences) as described in chapter 2. For western blot a standard curve was prepared with serial dilutions of standardised sea bream recombinant TTR and was always run in parallel to plasma samples collected from experiments. TTR was quantified by densitometry using the software Quantity One (free trial- Bio-Rad, USA). Time did not permit all experimental samples to be analysed and this will be carried out in the future.

RNA extraction and cDNA synthesis

Total RNA was extracted from liver, pituitary and brain using TRI reagent (Sigma-Aldrich) according to the protocol provided. Extracted RNA was resuspended in 20-500 µl DEPC-treated water and the quantity and quality assessed by electrophoresis of 1 µg RNA in a 1 % agarose gel and by spectroscopy (Pharmacia Biotech Ultraspec 3000) at A_{260} and by determining the ratio at A_{260}/A_{280}. Prior to cDNA synthesis RNA was treated with DNase using DNA-free Kit (Ambion, UK) following the suppliers instructions. Synthesis of cDNA was carried out in a 20 µl final reaction volume using, 0.5 µg of DNase-treated RNA, 1 mM of dNTPs mixture, 1 µg of random hexamer primers (pd(N)$_6$, Amersham Biosciences) and sterile water (Sigma-Aldrich). The reaction was heated at 65 ºC for 5 min to heat-denature RNA and incubated on ice for 5 min before adding the final reaction components: 25 U of RNAguard RNase inhibitor (Amersham Biosciences), 40 U of MMLV RT (reverse transcriptase) and 1x RT buffer (Invitrogen, Carlsbad, CA, USA). 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.

Quantitative real-time RT-PCR

Real-time RT-PCR reaction was performed using "Taqman" technology and a Bio-Rad iCycler iQ system and software (Bior-Rad laboratories). The correct probes and primers (see table 1) for each gene analysed (sea bream TSH, deiodinases II and III, TTR and TR-β) were designed using the original sea bream sequences using Beacon Designer software (Premier Biosoft international). The probes were labelled at the 5'-end with FAM and at the 3'-end with TAMRA-6-FAM (Thermo). The optimal concentration of probe and reverse and forward primers was established using the following criteria, highest fluorescence, best log fit and linear phase and lowest possible cycle number. Primer and probe concentration selected are given in table 1. Real time RT-PCR reactions were performed using Eppendorf qPCR mastermix in a 25 µl reaction volume and approximately 20 pg of sample cDNA with the optimal primer and probe concentrations. The thermocycle utilised was 1.5 min at 95 ºC followed by 45 cycles of 15 seconds at 95 ºC followed by 1 minute at 60 ºC. Quantification was carried out using the standard curve method with serial dilutions (1/10 interval from 1 ng/µl to 1 fg/µl) of cDNA plasmid of the target genes in each experiment. To normalise the quantity of each gene, target amplicons obtained per reaction were divided by absolute values of 18s rRNA.

Table 1. Quantitative RT-PCR primers and Taqman probe sequences and respective optimized concentration (below primer sequence) used to analyse sea bream expression of genes: D2, D3, TSH, TRβ and 18S which was used as a standard to normalise expression analysis.

Gene	Sequence Forward Primer qPCR Concentration (nM)	Sequence Taqman probe qPCR Concentration (nM)	Sequence Reverse Primer qPCR Concentration (nM)
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
TSH	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

Histology

Tissue samples for histology were fixed in Bouin–Holland fixative for one week at room temperature with gentle agitation and decalcified in 0.5 M Ethylene diamine-tetracetic acid (EDTA; USB Corporation) pH 8.0. Samples were then washed in water to remove excess fixative and placed in 70 % ethanol, dehydrated in a graded series of ethanol (70 % →100 %) and embedded in paraffin using an automated tissue processor (LeicaTP1020, Leica). Serial saggital sections (5 µm) of embedded tissues were cut and mounted on glass slides coated with 3-Aminopropyltriethoxysilane (APES; Sigma-Aldrich). Standard Haematoxylin-Eosin coloration was performed on dewaxed and rehydrated sections at an interval of 5 sections in each larvae to visualize the thyroid gland. Briefly, tissues were immersed in Harris Haematoxylin for 30 seconds, washed in tap water and distilled water and immersed in Eosin for 30 seconds, washed in distilled water with a few drops of acetic acid and then tap water.

Stained sections were then dehydrated, cleared and mounted in DePX (Sigma-Aldrich). Thyroid tissue morphology was examined and presence or absence of colloid, vacuolation of colloid, follicular epithelial folding and size were registered. Morphometrical measurements were taken to establish number and size of follicles and the height of thyrocytes and their nucleus in 10 follicles per section in 3 individual fish from control and PTU1 treated fish. Measurements were always carried out on sections containing the hypohyal of the hyoid arch and the hypobranchial of the first branchial arch. Measurements taken from 4 cells located 90º from one another within each follicle, using the software ImageJ for digital image analyse. The ratio nucleus/cell height was determined.

Statistics

Unless mentioned, statistical analysis of the data was carried out by one-way analysis of variance (ANOVA) followed by Tukey multiple comparison test (HSD) when significant differences (considered at $p<0.05$) were found between treatments. The statistical software utilised was Sigma Stat version 3 (SPSS, Chicago, USA).

Results

Condition factor (K)

This parameter is usually used as comparative and theoretical measure of fish physiological well-being. Values obtained for condition factor of the fish before and after the treatments in each group are represented in fig. 1-A. Data analysis revealed no significant differences between treatment groups and the control, or within each group at the start and end of the experiment (t-test was used). Indicating fish well being was not seriously affected by the

treatments or by the stress derived from experiment conditions and manipulation. No mortality was observed during the experiment.

Total protein

Total protein plasma concentration values found for each of the experimental groups are represented in fig. 1-B. Comparison between all treatment groups and the control showed no statistically significant differences were found PTU1, PTU10 and DES. However, a slight but significant increase on total plasma protein concentration ($p<0.05$) was found in fish exposed to ioxynil.

Total plasma TH levels

Concentration of T_3 and T_4 in fish plasma was determined by RIA and results are represented in fig. 1-C and D respectively. Data analysis revealed that there were no significant differences in T_3 or plasma T_4 concentrations in experimental and control fish at the end of the experiment.

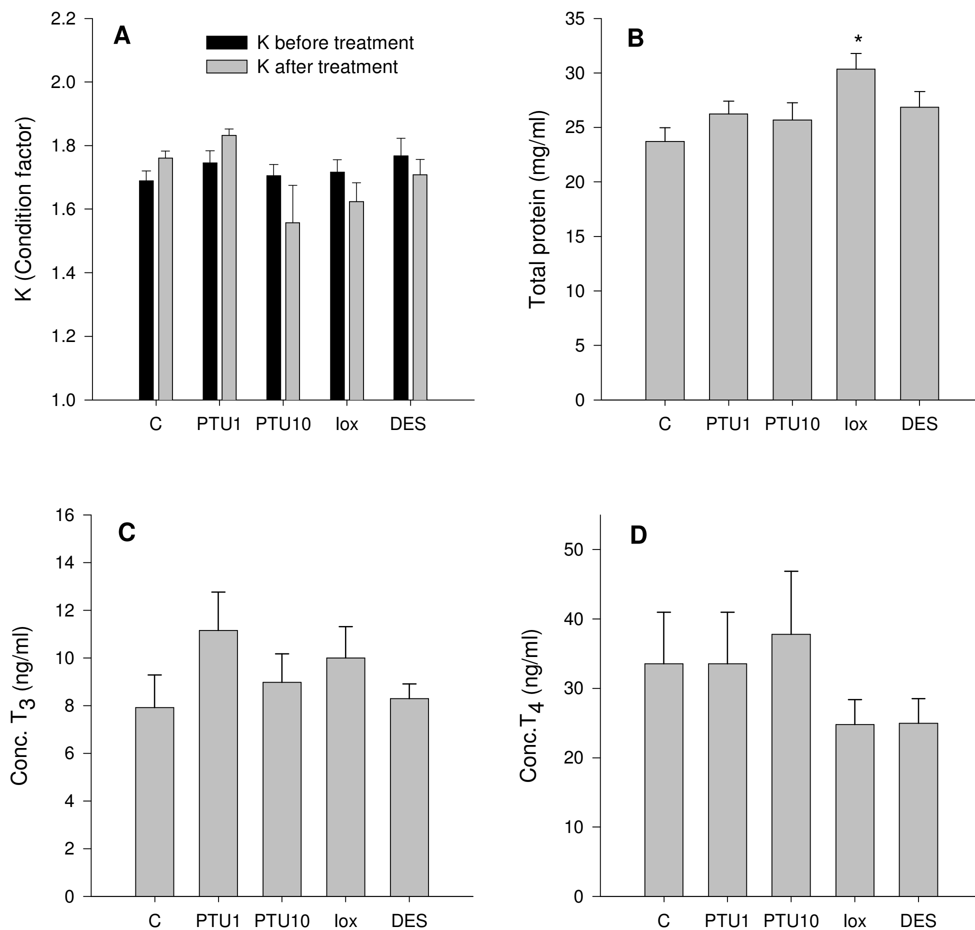

Figure 1. **A**- Changes in condition factor (K=100 x W x L^{-3}) of the fish before and after treatments: C- control group, PTU1- fish exposed to PTU at 1mg/kg fish, PTU10- fish exposed to PTU at 10 mg/kg fish, ioxynil- fish fed with ioxynil at 1mg/kg fish, DES- fish fed with DES at 1mg/kg fish. No significant alteration was found in *K* between the different groups and the control (p>0.05) at the end of the experiment or within the same group at the start and end of the experiment. **B**- Total plasma protein concentration (mg/ml) measured by Lowry assay in each group at the end of the experiment. Only ioxynil (p<0.05) caused a significant increase on total plasma protein. **C** and **D**- Concentration of plasma (ng/ml) of T_3 and T_4 levels, respectively, measured by RIA in each experimental group. No significant differences in T_3 and T_4 levels occurred between all the treatments and the control at the end of the experiment. Data correspond to mean ± SE (n=14 in A, C and D or n=10 in B) and differences were considered significant at p<0.05 (*).

Plasma TTR

An ELISA assay was previously developed (see chapter 5) to measure TTR plasma levels, however for technical reasons and because of lack of time the assay could not be used in the present study. An alternative technique, western blot coupled to chemiluminescence detection was utilised to measure plasma TTR, although only a small number of samples were analysed as a consequence of shortage of time. Preliminary analysis suggests that the treatments caused a modification in plasma TTR (fig. 2). The same sample volume (4 µl) of plasma from each fish was loaded in duplicate on gels for each group (except for PTU10 where only one sample was loaded). One principal band was detected by the anti-sbTTR sera. Visual analysis of bands intensity suggests a slight increase in TTR levels for ioxynil and PTU10 treated fish, and a strong increase in TTR levels for PTU1 group. In fish exposed to DES the strong intensity of the bands suggests a major elevation of TTR plasma levels in this group. A better quality image of a gel with 5 replicates for each group will allow confirmation and further quantification of these preliminary results.

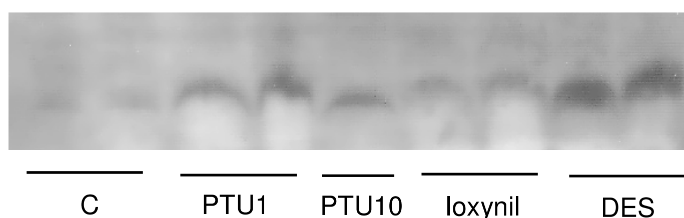

Figure 2. Western blot analysis with sbrTTR antisera (1:10000 dilution) of plasma samples from sea bream exposed to the different ED chemicals. The blot was developed by chemiluminescence and the autoradiograph was exposed for 40 min. Plasma from fish treated with vehicle alone C- control group; PTU 1mg/kg fish –PTU1; PTU 10mg/kg fish –PTU10; ioxynil 1mg/kg fish -Ioxynil; DES 1mg/kg fish -DES. The same sample volume (4 µl) of plasma from each fish was loaded on the gel in duplicate with the exception of PTU10 where only one sample was loaded.

Histology of thyroid follicles

Histological appearance of thyroid follicles was only analysed for fish exposed to the goitrogen PTU at 1mg/ml (group PTU1) as a consequence of shortage of time. Measurements of epithelial cell height and ratio nucleus/cell height revealed no significant difference from the control. However, the morphology of the follicles in PTU treated fish (fig. 3B) differs from that in the control fish (fig. 3A) as the follicle are of a more homogeneous size and appear to be flattened and the lumen is small and lacks colloid. The thyroid follicles in control fish contain abundant colloid with vacuoles present close to the thyrocyte interface and have a typical slightly oval appearance (fig. 3B). The observations in the control fish are characteristic of an active gland while those in the PTU treated fish are more typical of follicles with reduced activity.

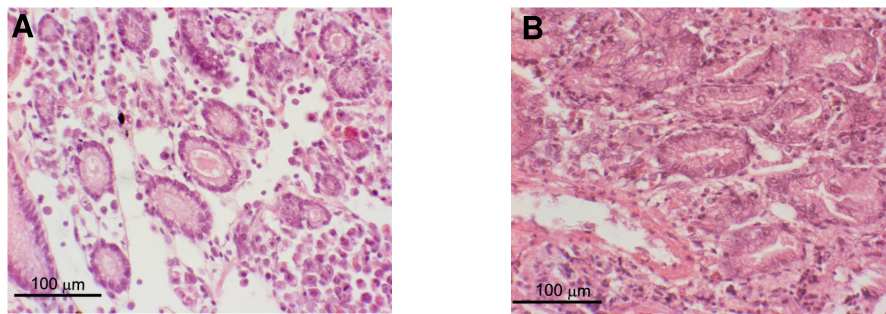

Figure 3. Haematoxylin and Eosin stained thyroid tissue from, **A**- control sea bream and **B**- PTU1 (1mg/kg fish). The thyroid follicles in the fish treated with the goitrogen PTU are flattened and contain scarce colloid. Amplification scale is indicated.

Effect of the different treatments on gene expression

The effect of the EDCs used on various elements of the sea bream thyroid system *in vivo*, was assessed by analysing target transcripts in different tissues by real-time RT-PCR. Transcript number for each gene was normalised by dividing by absolute values of 18s rRNA expression and are represented in relative units in fig 4. TSH expression was quantified in the pituitary

and brain and analysis showed that TSH expression levels in pituitary (fig. 4A) were significantly lower after exposure to 1mg/ml PTU ($p<0.001$), ioxynil ($p<0.005$) and DES ($p<0.001$) compared to control fish. Higher doses of PTU (10mg/ml) did not cause a significant decrease in TSH transcript number. All the experimental treatments caused a significant down-regulation of TSH expression levels ($p<0.05$) in the brain (fig. 4B) compared to control fish. TRβ transcripts in the pituitary were down-regulated by PTU1 ($p<0.001$) and DES ($p<0.05$) but not by any of the other chemicals tested (fig. 4C). Expression of TRβ in the brain and liver was assessed but no transcripts were detected in fish treated with EDCs or in the control fish. Deiodinase type I (DI), type II (DII) and type III (DIII) transcript abundance was also quantified in the pituitary, brain and liver. It was not possible to detect DI transcripts in any of the tissue collected. Deiodinase type II (DII) transcript abundance in the pituitary was not significantly altered by EDCs (fig. 4D), but was significantly down-regulated in the brain (fig. 4E) by all the chemicals tested ($p<0.001$). In the liver (fig. 4F) treatment with PTU (1mg/kg fish) caused up-regulation in DII expression ($p<0.005$) and no change in DII expression was observed in the other treatment groups compared to the control fish. It was not possible to detect DIII transcripts in the pituitary or the liver (not shown) but in the brain (fig. 4G) all EDCs tested caused down-regulation in DIII transcript number ($p<0.001$) compared to the brain in control fish.

A number of previous studies have demonstrated that TTR expression is principally restricted to the liver in fish and for this reason only this tissue was analysed (fig. 4H). Treatment with PTU (1mg/kg fish) significantly up-regulated TTR expression ($p<0.05$) compared to control fish liver. No significant changes in TTR transcript abundance was observed in other EDCs treatment groups compared to the control fish.

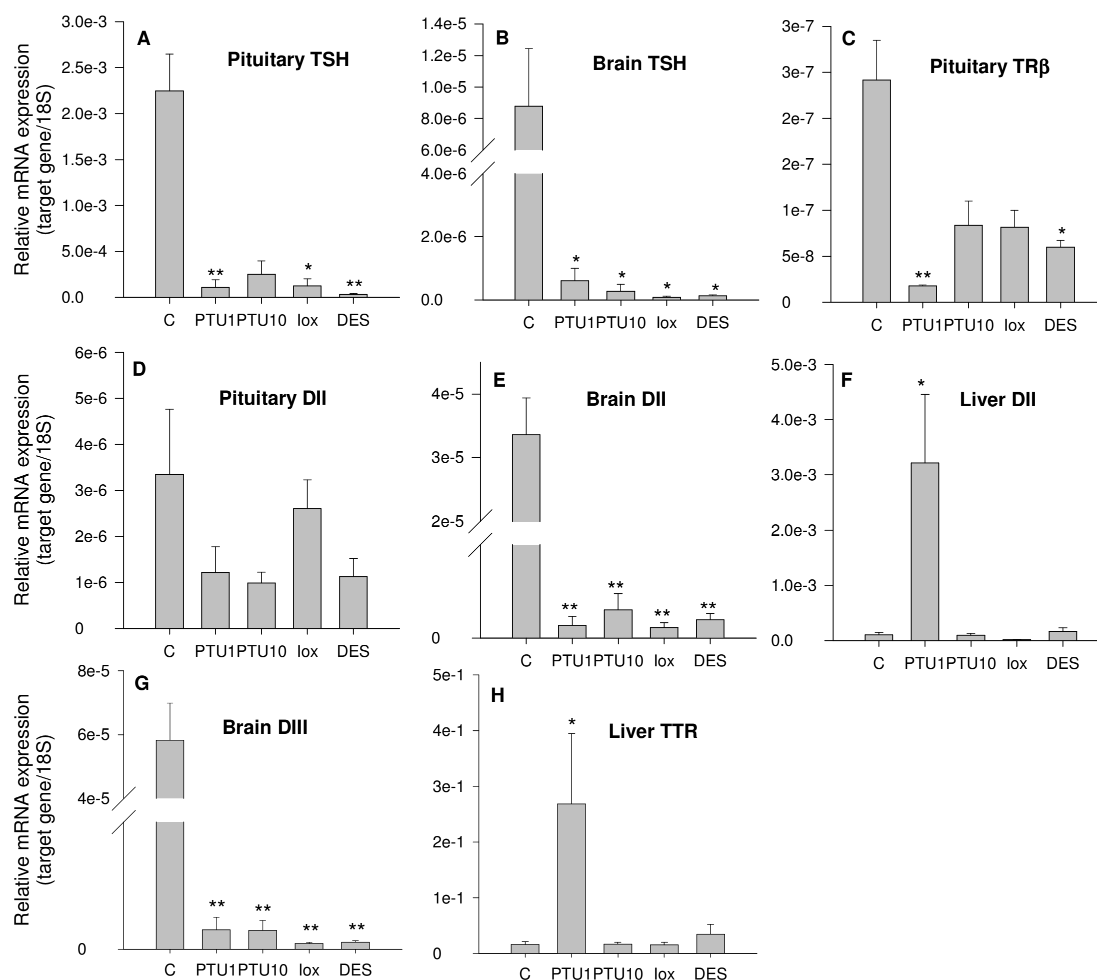

Figure 4. Quantitative real-time RT-PCR analysis of gene expression in fish exposed to different ED chemicals: C- control group, PTU1- PTU treatment 1mg/kg fish, PTU10- PTU treatment 10mg/kg fish, Iox- ioxynil treatment 1mg/kg fish, DES- DES treatment 1mg/kg fish. The values obtained for transcript number in the different treatments were normalised by dividing by absolute values of 18s rRNA expression values and are represented in relative units. Gene transcripts analysed for each treatment group are: TSH in pituitary and brain (A and B respectively); TRβ in pituitary (C); deiodinase type II (DII) in pituitary, brain and liver (D, E and F respectively); deiodinase type III (DIII) in brain (G); TTR in the liver (H). Data are presented as mean ± SE (n=6 or 5). Significant differences from corresponding controls are marked with * ($p<0.05$) and **($p<0.001$).

Discussion

Endocrine disrupting chemicals affect the thyroid system at different sites and can directly interfere with TH synthesis, TH metabolism and TH serum transport. They may also bind to TH receptors but data to support this hypothesis is still scarce (DeVito et al., 1999) and it has been suggested that TR may be a less important target for EDCs (Cheek et al., 1999; Ishihara et al., 2003b; van den Berg, 1990). Alteration in thyroid function is frequently determined by measuring TH serum levels as they give a fairly good indication of the central thyroid axis activity. The plasma THs act at the pituitary gland to cause positive or negative feedback on the release of TSH. A reduction in circulating TH levels leads to positive feedback and an increase in TSH secretion by the pituitary and if this is prolonged hypertrophy and hyperplasia of the thyroid tissue may occur in order to increase synthesis and release of THs. Exogenous EDCs may interfere with the metabolism of THs by modifying deiodinase enzyme activity or abundance and in this way modify hormone concentrations in serum and tissue. In most vertebrates, including teleosts, there are three types of deiodinases: type I (DI) which is found in liver, pituitary, thyroid, etc, which catalyses activation or inactivation of both T_4 and T_3 and their metabolites; type II (DII) which is mainly present in brain although in teleosts the liver also has a high activity (Orozco and Valverde-R, 2005) and low levels are also present in the CNS and pituitary. This enzyme has an essential role during hypothyroidism as it catalyses T_3 synthesis and is a major activating enzyme; and type III (DIII) which is present in brain and fetal tissue and is the major inactivating enzyme and catalyses the production of inactive rT_3 and 3,3'T_2.

The EDCs selected in the present study, DES and Ioxynil were chosen because *in vitro* (chapter 5) they had a high affinity for sbrTTR (a major THBP in fish) and might be expected to disrupt TH transport. Binding of exogenous chemicals to TTR *in vivo* may have a twofold effect: decrease affective free TH concentrations in plasma and consequently cellular uptake

and biological responses but also, displace THs from THBPs altering plasma TH homeostasis (Ishihara et al., 2003b). The overall consequence of EDCs binding to TTR may therefore results from the combined effect of these two situations. It has been hypothesized that TH displacement from THBP increases free TH clearance from plasma and decreases their circulatory concentrations (DeVito et al., 1999). It has also been suggested that TTR binding can be predictive of interactions with other proteins in thyroid system such as the deiodinases (Calvo et al., 1990) but this has never been assessed in fish.

Ioxynil and DES are chemicals that have high affinity for avian and amphibian TTR *in vitro* (Ishihara et al., 2003a; Ishihara et al., 2003b) and also (particularly ioxynil) sea bream TTR (chapter 5). Here we report that after 21 days of exposure to ioxynil (1 mg/kg fish) sea bream had slightly increased serum total protein levels. This might reflect an increase in levels of albumin or other plasma proteins and represent an acute response to toxicity. Preliminary analysis suggest that TTR plasma levels are significantly changed with all of the EDCs utilised in the study, although they did not modify TTR gene transcription in the liver (fig. 4H). Further analysis will be required before firm conclusions can be drawn, however, the mismatch between hepatic transcription and secretion is consistent with our previous observation (chapter 4) pointing to control of TTR at the level of secretion.

In fish treated with EDCs, levels of serum total THs were not significantly different from the control group in any of the treatments at the end of the experiment. Surprisingly, a highly significant down-regulation in TSH transcripts occurred in the brain and pituitary of all the experimental fish and may indicate EDCs act directly to influence TSH transcription in the brain and pituitary. The histological appearance of the thyroid tissue in fish exposed to PTU (1mg/kg fish) was indicative of a gland with reduced activity. It will be important to analyse the thyroid tissue in the fish exposed to the other EDCs. The apparently euthyroid status of fish despite down-regulation of TSH transcripts in fish treated with EDCs remains to be

explained but possible explanations may be the maintenance of normal levels of circulating TSH or other compensatory mechanisms which exist in the thyroid axis. Moreover, in the present study THs were analysed at a single time point and although initially chemical treatment may have disturbed homeostasis by the end of the experiment it may have been re-established. In favour of this suggestion are the results of previous studies in fish which report unaltered TH levels in response to EDCs but with changes in other thyroid axis endpoints (Brown et al., 2004).

All EDCs treatments affected TH metabolism in the brain and both DII and DIII expression levels were significantly depressed. In contrast, DII transcription was not affected in the pituitary or in liver (except by PTU1). Deiodinase activity is critical for regulation of TH tissue concentration and is strongly affected by TH serum concentrations in fish (Eales et al., 1993; MacLatchy, 1993; Mol et al., 1999; Orozco and Valverde-R, 2005). Tissue specific alterations in deiodinase activity have previously been observed in euthyroid fish after exposure to EDCs (for review see Brown et al (2004)) and it has been proposed that in fish EDCs induced changes in deiodinase activity may represent a compensatory mechanism to help preserve the euthyroid state. The modification in deiodinase observed in EDCs treatments in the present study fits well with the general notion that in teleost fish peripheral deiodination is the primary control mechanism of thyroid function, independent of the hypothalamo-pituitary-thyroid axis (Eales and Brown, 1993; Orozco and Valverde-R, 2005; Power et al., 2001). The difference in the relative importance of peripheral and central control of the TH axis in fish and mammals suggest that fish may have a more robust response to chemical disruption. It will be of interest in the future to assess the consequences of changing DII expression in the brain, particularly in relation to THBPs.

TRβ expression levels in the pituitary were not affected by exposure to ioxynil but treatment with DES seems to have caused a slight decrease in the number of receptor transcripts

suggesting suppression of TH responsive genes. Curiously, studies by Ishihara et al (2003b) also reported DES to be one of two EDCs showing some ability to bind the chicken and bullfrog TR ligand binding domain *in vitro*. Nevertheless, the great majority of studies suggest that few chemicals interfere with TRs (Cheek et al., 1999; Ishihara et al., 2003b) and there is generally a lack of evidence that EDCs bind to TR (DeVito et al., 1999).

PTU is a known anti-thyroid agent shown to have both intra- and extra-thyroidal anti-thyroid actions. In both animals and humans PTU inhibits many of the peripheral actions of THs and slows the peripheral deiodination (see Saberi et al (1975)). In fish PTU is known to have inhibitory effects on larval development (Liu and Chan, 2002; Osama and Klaus, 2003; Woodhead, 1966) and it has been reported to decrease TH levels in zebrafish (Wester et al., 2003). Here we tested two different doses of PTU: 1mg/kg fish (PTU1) and 10mg/kg fish (PTU10) as a positive control and for comparison to the effects of ioxynil and DES. Although unexpectedly none of the treatments altered TH levels at the end of the experiment. In fact, overall the effect of both PTU doses was comparable to the effects observed for DES and ioxynil on all the parameters studied and the only exception was the significant increase in DII and TTR expression in the liver caused by PTU1. The results suggest that neither DES nor ioxynil affected thyroid status more than the goitrogen PTU. It is tempting to speculate that binding of EDCs to TTR could decrease their cellular uptake and therefore biological consequences but much more work will be required to validate this hypothesis. The strong depression of deiodinase expression levels by PTU is consistent with its observed action in mammals. Again, such a decrease might reflect an effective compensatory peripheral mechanism which exists in fish to re-establish TH homeostasis. Analysis of thyroid tissue in the present study was only carried out for PTU1 (analysis is ongoing for other samples) and was consistent with a slightly less active gland. This is in accordance with the decreased TSH expression levels encountered in the pituitary which would lead to a reduction in TH

production. The enzyme DI is known to be particularly resistant to PTU in fish (Orozco and Valverde-R, 2005) and evaluation of the response of DI to PTU would be of interest but it was not possible to detect this enzyme in control or EDCs exposed fish. The fact that the lowest PTU (1mg/kg fish) dose was more effective than the higher dose (10mg/kg fish) in provoking changes in several elements of the thyroid axis is difficult to explain but may be related to the changing toxicity with time and accumulated dose during the experiment. Moreover, in general goitrogen effects are poorly understood and studied in all age classes of fish and further studies addressing this question are required.

In the present study two chemicals were selected for their strong TTR binding capacity *in vitro* and their possible *in vivo* consequences assessed in the sea bream. The chemicals tested appeared to have a strong effect on peripheral deiodination and the results suggest that the fish thyroid axis may have the ability to overcome disruption and re-establish TH homeostasis. The study is however, incomplete and further evaluation of thyroid histology and TTR plasma level quantification are currently being undertaken in order to better describe the endocrine disrupting effect of the test chemicals. It will be of interest in future to establish the relationship between EDCs which bind strongly to TTR *in vitro* and their *in vivo* consequences. The potential endocrine disrupting capacity of the test chemicals, a pesticide (ioxynil) and a synthetic estrogen (DES) is very relevant, as both are present in environmental effluents but relatively few studies on their influence on fish thyroid status exist (Brown et al., 2004).

Acknowledgements

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General Discussion

Transthyretin is a well characterised protein in higher vertebrates. It is a plasma protein involved in the transport of thyroid hormones and vitamin A. TTR association with severe human amyloidopathies is the object of intensive research. The strong affinity of TTR for putative endocrine disruptors could compromise thyroid system homeostasis although the importance of TTR's in thyroid axis regulation in not clear. In fish TTR has only recently been identified and relatively little is known about its function, regulation and interaction with THs and endocrine disruptors and the aim of this thesis was to investigate these questions. A recombinant TTR from the teleost sea bream (sbrTTR) was produced using a bacterial expression system. This approach was taken in order to generate sufficient quantities of TTR for experiments as its purification from fish plasma would be unlikely to generate the sufficient amounts. Bacteria expression systems are widely used to purify and characterize eukaryotic proteins as they have rapid growth, are easy to transform and induce, they are low cost and give high protein yields (Higgins and Hames, 1999). However, the major drawback of such systems is their inability to perform post-translational modifications typical of eukaryotes and the accumulation of proteins as insoluble inclusion bodies, in most cases due to protein misfolding (Sorensen and Mortensen, 2005). These issues were not a drawback for recombinant sbTTR production as demonstrated in previous studies of this protein (Eneqvist et al., 2004; Eneqvist et al., 2003). Furthermore, as one objective of the thesis was to evaluate the influence of the N-terminal on ligand binding the generation of mutant recombinant proteins is relatively straight forward. Purification of the recombinant TTR produced using native PAGE was sufficient for it to retain TH binding ability (and therefore its native conformation). Studies to ascertain protein native structural characteristics are also currently being performed using chromatography (HPLC) and circular dichroism (CD).

The binding affinity of sbrTTR to THs was established using an *in vitro* competitive binding assay and indicate it has a similar affinity for T_3 and T_4. However current theories suggest that

in lower vertebrates TTR was a T_3 binder and during evolution became a T_4 binder in eutherian mammals in association with the proposed evolution of T_4 as a pro-hormone (Schreiber, 2002a; 2002b). In fish there are few studies of TTR hormone affinity and those that exist are qualitative and indicate a slightly higher affinity for T_3 (Eneqvist et al., 2003; Santos and Power, 1999; Yamauchi et al., 1999). In our studies we developed and optimized a specific competitive binding assay for sbrTTR binding to THs which gives consistent and reliable results. Interestingly the assay conditions for piscine and human TTR binding assays differ significantly and suggest assay conditions are crucial for appropriate binding and may explain the very variable Kd obtained by different authors for the same proteins (for example see Chang et al (1999)). Further studies on TTR:TH binding from representative of the main classes of fish would be of great interest and the fact that TTR cDNA from the lamprey (an ancient species) was recently cloned (Manzon et al., 2007) opens interesting perspectives.

The proposed hypothesis that the length and hydrophilic nature of the N-terminus of TTR is important for TH binding properties was also explored. Two sbrTTR mutants were produced and it was observed that removal of the first 12 N-terminal (sbrTTRM12) amino acid does not alter sbrTTR:TH binding properties but when only the first 6 amino acids (sbrTTRM6) are removed the protein losses affinity for T_4. Therefore, the N-terminus which is thought to be placed at the entrance of the binding channel, appears to influence TTR:THs binding affinity and this was also reported by Prapunpoj et al (2006; 2002). It appears that the length *per si* of TTR may not be important but rather the physicochemical properties of the amino acid residues in this region. Possible interactions of the N-terminal residues with other residues at the binding site or even possible changes in conformation induced in these regions could be at the origin of the observed affinity changes. It is also noticeable that changes in affinity were only found for T_4 and this agrees with the observation of Prapunpoj et al (2006) who suggest the N-terminus exerts greater influence on the affinity of T_4 then that of T_3.

Fluorimetric measurements of sbrTTR in acidic conditions in order to evaluate fibril formation revealed that the mutant lacking the first 6 N-terminal amino acids (M6) which lost affinity for T_4 also failed to form amyloid fibrils. It is known that fibril formation can be prevented by stabilizing TTR tetramer at the binding site (Colon and Kelly, 1992; Miroy et al., 1996) and we therefore hypothesise that the M6 mutation either by interaction of local residues or conformational changes stabilized the tetramer. The work reported in the thesis presents for the first time the assessment of fibril formation by piscine TTR using spectroscopy and indicated that sbrTTR seems to be more resistant to this process compared to the human protein as was also suggested by Eneqvist et al (2003). The results about TTR stability are of considerable interest and open new perspectives and highlight the potential utility of comparative analysis in understanding TTR stability.

In the present work for the first time TTR levels in fish plasma were measured using a sensitive and specific ELISA assay developed for sea bream TTR. Using the assay it was possible to establish that TH:TTR ratio in fish is much higher than in humans and this may be related to species specific differences in THBP abundance and affinities or to a broader action TTR in humans beyond TH transport (e.g., RBP-retinol transport). Our experiments also indicate that THs appear to be involved in the regulation of plasma TTR levels, probably by changing its rate of secretion. As TTR appears to be the main THBP in fish it may have a fundamental role in regulating homeostasis of free levels of THs and when hormone levels increase it probably counteracts partitioning of free THs into cells. This is the first time that the regulation of TTR by THs has been considered in fish and in general many more studies are required to explore regulatory mechanisms in fish but also in other vertebrates. In contrast to mammals in which free T_3 levels are very low in fish plasma they are frequently similar to T_4 levels (Eales and Brown, 1993; Power et al., 2000b) and it is tempting to speculate that the progressive loss of affinity of TTR for T_3 during vertebrate evolution is associated with the

establishment of T_4 as a pro-hormone. Further comparative studies relating TH levels and TTR affinity in vertebrates and a better understanding of deiodinases system and the thyroid axis in fish would be of great interest.

The importance of TTR in TH transport in fish raises a number of questions about how it is affected by environmental chemicals which disrupt endocrine systems in humans and wild life. The majority of studies of endocrine disruptors are in the context of the reproductive system; however there is increasing evidence from animal and *in vitro* studies that the thyroid axis is also vulnerable to endocrine disruption. Thyroid hormone transport is an important endpoint of the thyroid cascade and a number of recent studies have indicated that TTR from vertebrates has high affinity for many exogenous chemicals (Cheek et al., 1999; Hamers et al., 2006; Ishihara et al., 2003a; Ishihara et al., 2003b; Lans et al., 1993; Rickenbacher et al., 1986; Yamauchi et al., 2000). In the context of the differing affinity of piscine TTR for THs and the frequent reports of water contamination by EDCs an *in vitro* study of a panel of putative EDCs was conducted using the competitive binding assay developed for sbrTTR. Chemicals from different sources (industrial, pharmaceutical and agricultural) and classes reported as environmental pollutants in aquatic environments (Anderson and MacRae, 2006; Brown et al., 2004), some of which accumulate (Akutsu et al., 2001; Manchester-Neesvig et al., 2001) or cause thyroid disruption in fish and other species were selected (Brown et al., 2004; Brucker-Davis, 1998). Some of the tested chemicals bound sbrTTR with similar or higher affinity than T_3. The use of sbrTTR as opposed to plasma (Ishihara et al., 2003b) in competitive binding assays allows the specific effect of EDCs on TTR to be established and avoids possible non-specific effects. The pattern of binding preferences of sbrTTR for EDCs was quite distinct from that of TTR from other vertebrates (Hamers et al., 2006; Ishihara et al., 2003a; Ishihara et al., 2003b) and are coherent with the different TH affinity found for this protein in fish (chapter 3). As piscine TTR seems to have quite distinct binding properties

from other vertebrates it is proposed that the developed piscine competitive binding assay represents a specific tool to assess potential thyroid axis disrupting by EDCs in fish. As fish are frequently used as sentinel species for quality of aquatic environments this would be a more accurate tool than previously proposed methods using TTR from different species (Yamauchi et al., 2003). Moreover, it would permit new chemicals to be tested for affinity to sbrTTR and provide indication of potential disruptive effects and ability to stabilize the native tetramer and inhibit fibril formation as previously proposed (Maia et al., 2005; Miroy et al., 1996; Morais-de-Sa et al., 2004). The *in vitro* binding assay can only be a preliminary risk assessment tool as poor/high binding affinity found for compounds *in vitro* does not directly indicate disrupting activity in the more complex *in vivo* system.

The identification of several chemicals with high affinity for sbrTTR *in vitro* led to the next study which was to try and relate this character to *in vivo* consequences. Brominated flame retardants like the tetrabromodiphenylether BDE-49 or tetrabromobisphenol A (TBBPA) which showed remarkable affinity to sbrTTR (chapter 5) or the commercial Bromkal 70-5DE which include BDE-47, 99 and 100, strong sbrTTR binders were not selected, as such flame retardants have been banned in USA and Europe (De Wit, 2000; Kucher and Purvis, 2004) and are not available in sufficient quantities. Instead ioxynil (herbicide, (Geiger et al., 1990)) and DES (synthetic estrogen, (Wenzel, 1995)) which bind to sbrTTR and TTR from other species (Ishihara et al., 2003a; Ishihara et al., 2003b) and are readily available were used *in vivo*. Such EDCs and their conjugated metabolites are found in wastewater or activated sludge. The chemicals are practically insoluble in water and therefore in the bioassays conducted they were administered via the diet rather than in tank water. Moreover, natural exposure of fish to EDCs through diet has commonly been reported in the literature (Brown et al., 2004; Brown et al., 2002; Coimbra et al., 2005) and is less invasive or stressful than administration through injection. No literature is available about the typical exposure levels

for the EDCs tested and the dose selected (1mg/kg fish accumulative dose over 21 days) reflected that reported for other EDCs (Brown et al., 2004; Coimbra et al., 2005).

No strong conclusion can be drawn from the study as it was not possible to complete sample analysis prior to the conclusion of this thesis, but it is clear that the administered chemicals influenced the thyroid axis. An interesting observation was that although TH levels were normal peripheral deiodination decreased (as assessed by reduction in deiodinase transcripts) supporting suggestions that in fish peripheral deiodination may control thyroid function rather than the hypothalamo-pituitary-thyroid axis (Eales and Brown, 1993; Orozco and Valverde-R, 2005; Power et al., 2001). The overall effect of PTU on the thyroid axis was greater than the EDCs and may indicate their binding to TTR may protect cells from their effects, although more studies will be required to test this hypothesis. Future studies of TTR plasma levels in fish exposed to EDCs will be crucial as will quantification/detection of TTR expression in the brain where the most significant changes in TSH and deiodinases gene expression were identified. It is not yet clear if in fish TTR regulates TH uptake into the brain (Santos et al., 2002; Schreiber, 2002b) but if it does changes in its expression caused by EDCs might explain the changes in TSH and deiodinases in this organ. Further studies will be required to establish if correlations exist between TTR binding to EDCs *in vitro* and their effect *in vivo* in fish.

The work reported in this thesis gives important insights into thyroid hormone transport in fish and their regulation, TTR structure/function evolution and amyloid properties and the ability and consequences of TTR binding to putative EDCs. In addition to attaining the objectives initially proposed a number of new and interesting questions arose from the results of the work. The tools developed will be important for future studies aimed at understanding the role of THBP in fish and other vertebrates and factors which determine binding affinity and stability of TTR. The TTR binding assay could be used to identify new molecules with

the ability to bind and stabilize the native tetramer and inhibit fibril formation. Moreover, the preliminary studies on endocrine disruption and the possible role of TTR indicate that this will be a fruitful area of research and emphasizes the need for future studies and should provide insights into the thyroid axis in fish.

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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.

Solutions used in plasmid DNA purification by Alkaline Lysis

GTE (Glucose-Tris-EDTA) buffer
50 mM glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0. Autoclave.

3M KAc
60 ml of 5M potassium acetate (KAc)
11.5 ml of glacial acetic acid
Add dH_2O to a final volume of 100 ml and autoclave.

Solutions used for SDS-PAGE and Native 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.

Coomassie blue staining solution
0.25% (w/v) Coomassie brilliant blue, 10% (v/v) glacial acetic acid, 45% (v/v) methanol.

Destain solution
10% (v/v) glacial acetic acid, 10%(v/v) methanol.

Silver nitrate staining solutions

Staining solution: 21ml of NaOH 0.36%, 1.4ml of NH_4OH 29.5%, 4ml of a 0.2 g/ml $AgNO_3$ solution. Add the $AgNO_3$ to the solution drop by drop with vigorous agitation. Adjust the final volume to 100ml with MillQ H2O

Developing solution: 0.5 ml of 1% (v/v) citric acid ($C_6H_8O_7$), 0.05 ml of formaldehyde 38% and MilliQ H2O to 100ml

Stop solution: 100ml of 1% acetic acid in MilliQ H2O

Glycine-acetate 1X running buffer
Glycine 0.2M; Sodium Acetate 0.12 M

Glycine-acetate gel 12.5%

Reagents	Gel (Total volume = 4 mL)
Polyacrylamide percentage	**12.5%**
ddH_2O	8.37 ml
40%Acrylamide:Bisacrylamide	4.7 ml
Gly-Acet 5x buffer	3.04 ml
10% Ammonium Persulfate (APS)	0.375ml
TEMED (N,N,N',N'-Tetramethylethylenediamine)	0.015 ml

<u>Solution for Western blot</u>

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.

<u>Prep cell preparative SDS-PAGE</u>

<u>Resolving gel</u>: (internal diameter 28mm with 3.6 cm^2 surface area, 20mL): 15% polyacrylamide in 0.375 M Tris-HCl, pH 8.8.
<u>Stacking gel:</u> (same as resolving, 10mL) - 4% polyacrylamide in 0.125 M Tris-HCl, pH 6.8.
<u>Sample buffer</u>: 0.06 M Tris-HCl pH 6.8; 0.025% bromophenol blue; 2% SDS, 10% glycerol
<u>Running buffer</u>: 0.025 M Tris-HCl, 0.192M glycine buffer, pH 8.3
<u>Running time</u>: approximately 10h
<u>Sample volume</u>: ~1.2 ml

Gel preparation:

Reagents	Stacking Gel (Total volume = 10 mL)	Resolving Gel (Total volume = 20 mL)
Polyacrylamide percentage	**4%**	**15%**
ddH$_2$O	6.43 ml	7.4ml
40%Acrylamide:Bisacrylamide*	1 ml	7.5ml
Tris-HCl buffer	2.5ml of 0.5M Tris-HCl pH6.8†	5ml of 1.5M Tris pH 8.8‡
10% Ammonium Persulfate (APS)	0.05 ml	0.05 ml
TEMED (N,N,N',N'-Tetramethylethylenediamine)	0.01ml	0.005ml

* PlusOne ReadySol IEF acrylamide solution with 3% bisacrylamide (Amersham Biosciences)
†**0.5 M Tris, pH 6.8:** 6.05 g of Tris-base (Merck) in 75 ml ddH$_2$O, adjust pH to 6.8 with concentrated HCl and add ddH$_2$O to 100 ml.
‡**1.5 M Tris, pH 8.8:** 36.2 g of Tris-base (Merck) in 150 ml ddH$_2$O, adjust pH to 8.8 with concentrated HCl and add ddH$_2$O to 200 ml.
Degas gels for approximately 30 minutes before adding APS and TEMED

Prep cell preparative large scale Native-PAGE

Resolving gel - (internal diameter 37mm with 8.2 cm^2 surface area, 60mL): 10% polyacrylamide in 0.375 M Tris-HCl, pH 8.8.
Stacking gel (same as resolving, 20mL) was of 4% polyacrylamde in 0.125 M Tris-HCl, pH 6.8.
Sample buffer - 0.0625 M Tris-HCl pH 6.8; 0.025% bromophenol blue; 10% glycerol
Running buffer: 0.025 M Tris-HCl, 0.192M glycine buffer, pH 8.3
Running time: approximately 7h
Sample volume: ~8 ml

Gel preparation:

Reagents	Stacking Gel (Total volume = 20 mL)	Resolving Gel (Total volume = 60mL)
Polyacrylamide percentage	**4%**	**10%**
ddH$_2$O	12.86 ml	29.94ml
40%Acrylamide:Bisacrylamide	2 ml	14,94ml
Tris-HCl buffer	5ml of 0.5M Tris-HCl pH6.8†	15ml of 1.5M Tris pH 8.8‡
10% Ammonium Persulfate (APS)	0.1 ml	0.150 ml
TEMED (N,N,N',N'-Tetramethylethylenediamine)	0.02ml	0.015ml

† ‡ see the upper table

Solutions for the binding assay

TCN buffer
20mM Tris/HCl, pH 7.5, 93mM NaCl, 1mM $CaCl_2$ and 1mM $MgCl_2$ in ddH20

bovine γ-globulin
125μg/ml

Separating solution
25% (w/v) and PEG 6000 containing 0.2M $ZnCl_2$ in TCN buffer

Washing solution
12.5% (w/v) PEG 6000 containing 0.1M $ZnCl_2$ in TCN buffer

Culture media

LB (Luria-Bertani) broth
Dissolve 1 LB broth tablet (Sigma) / 50 ml dH_2O and autoclave. Final composition for 1 liter: 5 g NaCl, 10 g tryptone, 5 g yeast extract. For LB broth with antibiotics, add antibiotic to the desired concentration (see below) after autoclaving and store at 4ºC.

LB agar plates
Dissolve 1 LB agar tablet (Sigma) / 50 ml dH_2O, autoclave, allow to cool to 55ºC and pour into Petri dishes. Final composition for 1 litre: 5 g NaCl, 10 g tryptone, 5 g yeast extract, 15 g agar. For selective agar plates, add antibiotic, IPTG or X-Gal to the desired concentration before pouring into petri dishes.

Antibiotics, IPTG and X-Gal:

Ampicillin	Stock 50 mg/ml	Used at 80-100 μg/ml
Chloramphenicol	Stock 34 mg/ml	Used at 34 μg/ml
Kanamycin	Stock 50 mg/ml	Used at 50 μg/ml
IPTG	Stock 0.5M	Used at 0.5 mM
X-Gal	Stock 80 mg/ml	Used at 80 μg/ml

Primers (5'→ 3')

sbTTRWT
fw TTT TTC ATG ACC CCC ACC CCC ACG
rv TTT CGA GCT CAC TCG TGT ACG CTG GAG

sbTTRM12
fw **GCT AGC** TGT CCT CTG ATG GTA AAA ATC C
rv GGA TCC TCA CTC GTG TAC GCT GGA GAC

sbTTRM6
fw **GCT AGC** GGA GGC TCA GAC ACC AGG TG
rv GGA TCC TCA CTC GTG TAC GCT GGA GAC