Universidade do Algarve

Faculdade de Engenharia de Recursos Naturais

Thyroid Axis disruption by goitrogens: a molecular and functional approach

Dissertação de Mestrado Integrado em Engenharia Biológica

Eduarda Mazagão Guerreiro

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Dissertação orientada por
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I. Abstract

The number of chemicals to which organisms are exposed as a consequence of environmental contamination by Industry and Agriculture is increasing. However, there is a severe lack of information about the biological effects of many frequently occurring chemicals.

The present thesis is focused on the effects on the thyroid tissue of adult zebra fish of the antimicrobial agent Triclosan (TCS) which is frequently found in personnel hygiene products and foodstuff. After 21 days exposure of adult zebrafish to TCS (100 mg/kg/day) and the thyroid disruptor, Propylthiouracil (PTU, positive control) (5 mg/kg/day), histological observation of thyroid tissue in sections of the pharynx was performed. These observations indicated significant ($p<0.05$) increase in follicle areas coupled with the decrease of thyrocyte height (thyrocyte inactivation) in TCS treated zebrafish. In the PTU treated zebrafish only a reduction in thyrocyte activity was observed.

In order to understand how the test chemical TCS might bring about its effect, the gene expression of Thyroglobulin (Tg), Thyroid Peroxidase (TPO), Sodium-Iodine Symporter (NIS) and Cathepsin B (CtsB) together with Thyroid Stimulating Hormone (TSH) in whole zebrafish head was determined using quantitative real-time Polymerase Chain Reaction (qRT-PCR). After sample normalization against the zebrafish spermatogenic glyceraldehyde-3-phosphate dehydrogenases (GAPDH-2), validated as reference gene for this study, a significant increase ($p<0.05$) in expression of TSH and NIS were detected. The expression of pituitary TSH increased in the PTU and TCS groups compared to the control group, while NIS expression only increased in the TCS treated zebrafish. For the remaining genes, no significant changes in gene expression were detected, maybe as a consequence of high individual variation.

In summary, the results of the study indicate that the drug TCS at a concentration of 100 mg/kg/day for 21 days appears to influence TH synthesis. The increase in TSH and NIS transcription coupled to the inactivation of the thyroid tissue observed in this work, are indicative of effects caused by a reduction in circulating THs. It remains to be established the mechanism by which TCS reduces thyroid tissue activity in adult zebra fish.

Key works: Triclosan, Propylthiouracil, thyroid disruptor, follicle inactivation, qPCR, and relative expression.

II. Resumo

O número de químicos aos quais os organismos estão expostos diariamente por contaminação ambiental causada pela indústria e/ou agricultura tem vindo a aumentar. Há, no entanto, uma grande falha na informação sobre os efeitos biológicos destes contaminantes.

Esta tese foca-se nos efeitos do agente antimicrobiano Triclosan (TCS), no tecido da tiróide de peixe zebra adulto. Após 21 dias de exposição dos animais, ao TCS (100 mg/Kg/dia) e ao agente Propiltiouracil (PTU, controlo positivo) (5 mg/Kg/dia) foram efectuadas observações histológicas em secções da faringe. Estas observações indicaram um aumento significativo ($p<0.05$) no tamanho dos folículos assim como inactivação dos tirócitos no grupo exposto ao TCS e redução da actividade dos tirócitos nos peixes tratados com PTU.

Para compreender o modo de acção dos compostos, a expressão dos genes Tiroglobulina (Tg), Tiróide Peroxidase (TPO), Transportador Sódio-Iodo (NIS) e Catepsina B (CtsB), assim como Hormona Estimuladora da Tiróide (TSH), em toda a cabeça de peixe zebra foi estudada recorrendo à técnica de PCR quantitativo em tempo real (qRT-PCR). Após a normalização da amostra contra o gene gliceraldeido-3-fosfato dehidrogenase espermatogénico (GADPH-2), validado como gene referência neste estudo, detectou-se um aumento significativo ($p<0.05$) na expressão dos genes TSH e NIS. A expressão relativa da TSH aumentou nos grupos TCS e PTU, enquanto que a expressão do NIS mostrou aumento significativo apenas no grupo tratado com TCS. Nas restantes proteínas não foram detectadas alterações significativas das expressões possivelmente pela variação biológica entre os indivíduos.

Os resultados obtidos indicam que o composto TCS, com uma concentração de 100 mg/kg/dia por 21 dias, parece influenciar a síntese das hormonas da tiróide. O aumento da transcrição de TSH e NIS assim como a indução de inactividade do tecido da tiróide, observados neste trabalho, são efeitos indicativos da redução das hormonas da tiróide em circulação. É ainda necessário estudar qual o mecanismo através do qual o TCS reduz a actividade da tiróide nos peixes zebra adultos.

Palavras-chave: Triclosan, Propiltiouracil, hipotiroidismo, inactivação folicular, qPCR, expressão relativa.

III. Abbreviations

18 S	18 S ribosomal subunit
ANOVA	Analysis of variance
APES	Aminopropyltriethoxysilane
bp	Base pair
cDNA	Complementary DNA
Ct	Threshold cycle
CtsB	Cathepsin B
CtsBa	Cathepsin B, isoform a
DEPC	Diethylpyrocarbonate
DIT	Diiodotyrosine
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleotide triphosphate
ED	Endocrine disruptor
EDTA	Ethylenediaminetetraacetic acid
GAPDH-2	Spermatogenic glyceraldehyde-3-phophate dehydrogenase
gDNA	Genomic DNA
HPT axis	Hypothalamic-pituitary-thyroid axis
IPTG	Isopropyl-beta-D-thiogalactopyranoside
LB Broth	Luria-Bertani broth
MCE Group	Molecular and comparative endocrinology group
MIT	Monoiodotyrosine
MgCl2	Magnesium Cloride
MMLV-RT	Mouse moloney murine leukemia virus reverse transcriptase
mRNA	Messenger ribonucleic acid
NIS	Sodium-Iodine symporter
PCP	Personal care products
pDNA	Plasmid DNA
PFA	Paraformaldehyde
PTU	Propylthiouracil
qRT-PCR	Quantitative real-time Polymerase Chain Reaction
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Rotation per minute
RT-PCR	Reverse transcriptase – polymerase chain reaction
SDS	Sodium dodecyl sulphate
SEM	Standard error
SQ	Starting quantity
T3	Triiodothyronine
T4	Thyroxine
Ta	Annealing temperature
TCS	Triclosan
Tg	Thyroglobulin
TH	Thyroid hormone
Tm	Melting temperature
TPO	Thyroid peroxidase
TRH	Thyroptropin releasing hormone

TSH	Thyroid stimulating hormone
X-Gal	5-bromo-4-chloro-3indolyl-beta-D-galactopyranoside

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1. Introduction

1.1 The Endocrine System

The tissues and organs of the vertebrate body cooperate to maintain homeostasis of the body's internal environment, through the actions of multiple regulatory mechanisms that involve many organs of the body [1]. The **endocrine system** regulates many of these mechanisms.

The endocrine system sends information to the tissues using chemical substances released by the **endocrine glands**. These substances – **hormones** – are released into the circulatory system and transported to all parts of the body [2] thus establishing a complex interaction between organs, as represented in **figure 1.1**.

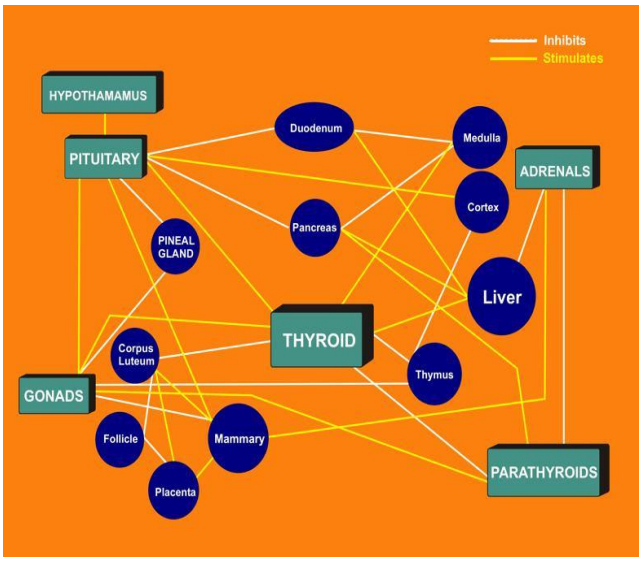

Fig 1.1: Schematic representation of the relationship of endocrine organs with the body tissues. As seen in this scheme of the endocrine system, the organs share a rather complex relationship. Taken from [3].

A hormone is traditionally defined as a substance that 1) is produced in small amounts by a set of cells in endocrine glands, 2) is secreted into the interstitial spaces, 3) enters into the circulatory system that carries it to other parts of the body, and 4) affects a limited number of cells, which are called **target cells** [2, 4]. However, it is now known that they may move by circulation in blood, other body fluids or diffuse between

cells, and may act in distant organs in the body (endocrine action) or the chemical regulatory molecules may be released and act within an organ regulating the same cell type which produced it (autocrine) or act in different tissues of the same organ (paracrine) [1].

The response of target cells to the hormone to which they are sensitive is due to the presence of hormone-specific receptors on the cells. These receptors might be found on the surface, **extracellular receptors**, or in the cytoplasm or nucleus of the cell, **intracellular receptors**. The type of hormone and the kind of receptors to which the hormone is associated, depends of the chemical nature of the hormone molecule.

1.2 Hormones

Nature uses a diverse spectrum of molecules as hormones, and, like all molecules, hormones are synthesized, exist in a biologically active state for a time, and then degrade or are destroyed [4].

Hormones belong to four different chemical categories:

1. **Polypeptides**. These hormones are composed of chains of amino acids (aa) that are shorter than about 100 aa.
2. **Proteins**. These are composed of a polypeptide significantly longer than 100 aa, that might be glycosylated.
3. **Amines**. These are derived from the aa tyrosine and tryptophan.
4. **Steroids**. These are lipidic hormones derived from cholesterol [1].

In a more general approach, hormones can be divided into those that are lipophilic (lipid soluble) and those that are polar (water-soluble). The lipophilic hormones – all of the steroid hormones and thyroid hormones – as well as other lipophilic regulatory molecules can easily enter cells through the lipid portion of the cell membrane. Water-soluble hormones, in contrast, cannot pass through cell membranes, and must regulate their target cells through different mechanisms at the cell surface [1].

1.2.1 Hormones with intracellular receptors

Hormones are found dissolved in plasma and are transported either in a free form or bound to plasma proteins [2] – **carrier proteins**, some being specific for a certain hormone. Lipophilic hormones are mainly transported bound to carrier proteins, as they do not dissolve in the plasma. When the hormones arrive at their target cells, they dissociate from carriers and diffuse through the plasma membrane. The hormone binds to specific intracellular receptors (the **nuclear receptors**) in the cytoplasm or the nucleus, and hormone-receptor complexes then act as ligand-dependent transcription factors in the nucleus, by binding to promoter regions of responsive genes and stimulating/repressing their transcription [1, 4]. The production of these responsive-genes and their protein products will then lead to the final effect of the hormone.

1.2.2 Hormones with extracellular receptors

Hormones that are too polar to cross the plasma membranes of their target cells include all of the peptide, amino acid derivatives and glycoprotein hormones. These hormones bind to receptor proteins located on the outer surface of the plasma membrane [1], initiating a series of events that are mediated by **second messenger** molecules [1, 4]. Second messengers include organic molecules and Ca^{2+}, and changes in their concentrations in response to hormone-receptor binding trigger a cascade of intracellular signalling responses, generally involving the activation of multiple effector enzymes such as protein kinases, which lead to the hormone's physiological effect.

The binding of a water-soluble hormone to its receptor is reversible and usually very brief. After the hormone binds to its receptor and activates a second-messenger system, it dissociates from the receptor and may travel in the blood to another target cell somewhere else in the body. Eventually enzymes (primarily in the liver) degrade the hormone by converting it into inactive derivates [1].

1.2.3 Control of Endocrine Activity

The physiologic effects of hormones depend largely on their concentration in blood and extracellular fluid. Thus, precise control over circulating concentration of hormones is therefore crucial [4].

The concentration of hormone that reaches target cells is determined by:

- *Rate of production*: the synthesis and secretion of hormones is the most highly regulated aspect of endocrine control. Such control is mediated by **positive** and **negative feedback** circuits.

- *Rate of delivery*: an example of this effect is blood flow to the target organ or to a group of target cells – high blood flow delivers more hormone than low blood flow.

- *Rate of degradation and elimination* [4].

Feedback circuits are at the root of most control mechanisms in physiology, and are particularly prominent in the endocrine system. Instances of positive feedback certainly occur, but negative feedback is much more common [4]. Negative feedback ensures that the cellular concentrations of products determine the rates of their formation, thus ensuring that the cell synthesizes only as much as it needs [5].

1.3 Thyroid hormones

Thyroid hormones (TH) are small liposoluble molecules with two bioactive forms, tetraiodo-L-thyronine (**thyroxine**, **T_4**) and triiodo-L-thyronine (**T_3**), which contain four or three iodine atoms respectively [6], as showed in **figure 1.2**.

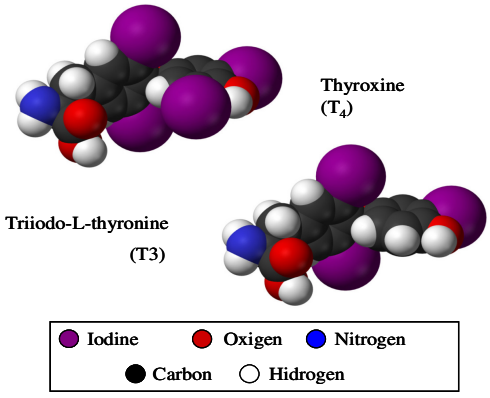

Fig 1.2: Molecular structure of the two thyroid hormone bioactive forms, thyroxine and triiodo-L-thyronine. Taken from [7].

THs stand out from all others present in vertebrates since they are the only iodine-containing compounds of physiologic significance [8].

THs are produced by all vertebrates, primarily as T_4 in the **thyroid gland** and are subsequently converted to the more bioactive T_3 form in peripheral tissues through the action of a family of enzymes the deiodinases [9].

These hormones are known to play a crucial role in maintenance of normal physiological functions [10] as they stimulate oxidative respiration in most cells in the body and, in doing so, help to set the body's basal metabolic rate [1].

THs are present in all vertebrates. For instance in children, they promote growth and stimulate maturation of the central nervous system, while in amphibians THs are needed for the metamorphosis of the larvae into adults [1]. In avian species, THs are required for nervous system and skeleton development [8].

In fish, TH action has also been demonstrated to regulate both larval and metamorphic development. It has also been showed that at later life stages in fish, THs assist in the control of various physiological functions including osmoregulation, metabolism, somatic growth, development, metamorphosis, etc. [11].

Although THs regulate many biological functions, their levels are also influenced by nutritional status in both endothermic (birds, mammals) and ectothermic (fish) vertebrates and poor nutrition is linked with a decreased T_3 and T_4 [8].

For the correct concentrations of THs to be maintained there are mechanisms which regulate THs synthesis. These mechanisms are found in the **Hypothalamic-Pituitary-Thyroid** (HPT) **Axis**, which, as the name indicates, consists of the hypothalamus, pituitary and the thyroid gland.

1.3.1 TH regulation

The activity of the thyroid gland, and all structures associated in the HPT axis, is predominantly regulated by the concentration of the pituitary glycoprotein hormone, **thyroid stimulation hormone** (**TSH**). Thus, regulation of thyroid function in normal individuals is to a large extent determined by the factors which regulate the synthesis and secretion of TSH. Those factors are mainly **thyrotropin releasing hormone**

(**TRH**), produced in the hypothalamus, and the feedback effects of circulating THs at the hypothalamic and pituitary levels of TRH and TSH ("thyrotropin") [8].

TRH is delivered to the pituitary gland and selectively stimulates the synthesis of the TSH beta subunit. Pituitary TSH is composed of two sub-units, alfa and beta [10]. The beta subunit confers specificity to the molecule, since it interacts with the thyroid cell TSH receptor leading, through enzymatic activity, to the increase in TH synthesis and liberation [8]. However, the TSH beta sub-unit in its free form is inactive, and requires non-covalent combination with the alfa subunit to express hormonal bioactivity [8].

TRH synthesis and release are regulated by the THs, an integral part of the negative feedback loop regulating thyroid status [8]. On the other hand, the major regulators of TSH production are represented by the inhibitory effects of thyroid hormones (negative feedback loop) and by the stimulatory action of TRH (**figure 1.3**) [8].

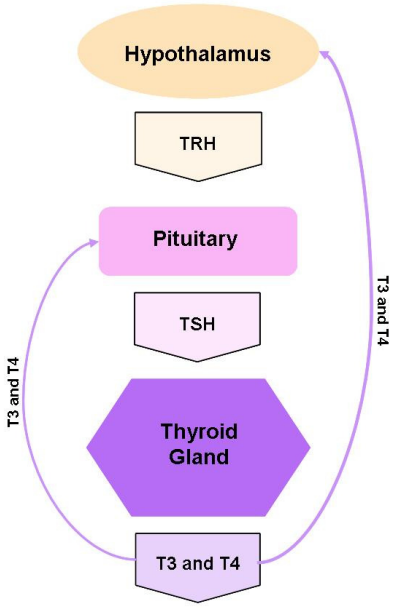

Fig 1.3: Fig. Schematic representation of the basic elements of the Hypothalamic-Pituitary-Thyroid (HPT) Axis: main glands and respective hormones. TRH is secreted by the hypothalamus and stimulates the pituitary to secret TSH, which then stimulates the thyroid to release T3 and T4. These hormones in turn will stimulate the target cells and regulate by negative feedback TRH and TSH synthesis.

1.3.2 Thyroid tissue

The thyroid tissue is composed of functional units called the **thyroid follicles**. The follicles are lined by a simple **epithelium** and their central cavity contains a gelatinous substance called **colloid** [12] **(figure 1.4)**.

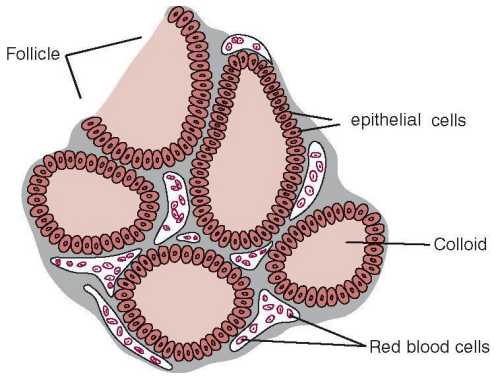

Fig 1.4: Representation of thyroid tissue showing the thyroid follicles containing colloid which is enclosed by a monolayer of epithelial cells. Taken from [13]**.**

The specialized epithelial cells found in the thyroid follicles concentrate iodide and incorporate it into **thyroglobulin** (Tg), which is subsequently hydrolysed to release THs. These processes require cell-type specific gene-products, which include Tg, **thyroid peroxidase** (TPO), the **receptor for the thyroid stimulating hormone** (RTSH) and **sodium-iodide symporter** [14], as explained below.

As mentioned in section 1.2.1, TSH has an important role in TH regulation. This hormone interacts with its receptor found in the thyroid cells which leads to transcription of the Tg, TPO, RTSH and NIS genes [10].

Although THs and thyroid tissue are present in all vertebrates there exist significant differences among species. In general, the gland is often a collection of aggregated follicles as described for mammals, highly vascularised and encapsulated by connective tissue [10]. However, the thyroid of many teleosts is not a compact single organ. They have the thyroid follicles loosely distributed within the mesenchyme of the ventral head area [11], although it maintains the tissue organization as thyroid follicles.

Despite these structural differences, the biochemistry and regulation of thyroid hormone synthesis are identical [10].

1.3.3 Hormone Synthesis

Synthesis and accumulation of THs takes place in four stages: synthesis of thyroglobulin, uptake of iodide from the blood, activation of iodide, and iodination of the tyrosine residues of thyroglobulin [12] (**figure 1.5**).

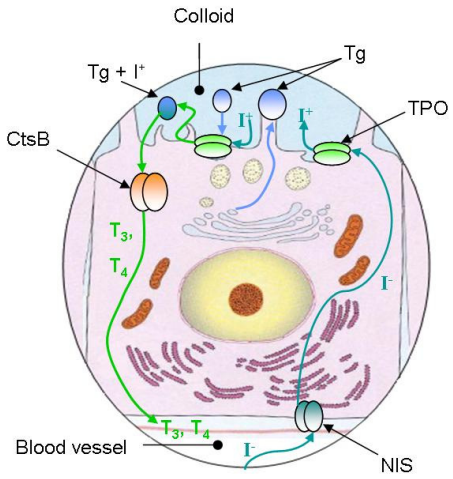

Fig 1.5: The thyroid follicular cell and the process of synthesis and secretion of THs. These events may occur simultaneously in the same cell. Taken from [12].

Synthesis of thyroglobulin – Tg is the substrate from which THs are synthesised [10]. Tg is secreted from the thyrocytes into the follicular lumen to form the colloid [15]. Tg is synthesised by ribosomes bound to the rough endoplasmatic reticulum and then transported to the Golgi apparatus, where carbohydrate moieties are added [10, 16]. Then Tg is released from vesicles which form at the apical surface of the cell into the lumen of the follicle where tyrosine residues are iodinated and where it is condensed to produce the thyroid hormones, T_3 and T_4. T_3 and T_4 remain covalently bound to Tg as long as they are stored in the lumen [12, 17].

Uptake of circulating iodide – is accomplished in the thyroid follicular cells [12]. The iodide uptake across the basolateral membrane of polarized cells is dependent of a specific transporter, the Na^+/I^- symporter or NIS. This is an intrinsic plasma membrane protein on the thyroid epithelial cells, structurally and functionally conserved among vertebrates [10, 18, 19], that passively transports two Na^+ and one I^- down the Na^+ ion gradient, resulting in an iodine concentration gradient from the thyroid cell to the extracellular fluid. The iodide gradient can be increased to as high as 1:400 in conditions of iodine deficiency [16]. Serum iodine plays an important role in regulating

thyroid function because low iodine levels increase the amount of NIS, and thus increase iodide uptake, compensating for the lower serum concentration [12].

Iodide oxidation – Iodide, the form of iodine that enters the cell, must be oxidized to a higher oxidation state before it is transferred to Tg. Oxidation of iodine is controlled by the enzyme thyroid peroxidise (TPO) [10], a membrane-bound glycoprotein with a central role in thyroid hormone synthesis catalysing iodide oxidation, iodination of tyrosine residues in Tg, and iodothyronine coupling [16]. Like Tg and NIS, the TPO enzyme is also highly conserved among vertebrates [10]. After the iodide is oxidised, another transport protein of the cell is involved, **pendrin**, that functions as an apical porter of iodide in the thyrocyte, transporting iodide into the follicle lumen [20].

Iodination of tyrosine residues – There are four major sites on the Tg protein where iodine becomes covalently attached. These "sites" are tyrosyl residues that accept an iodine atom as the consequence of TPO activity. Tg appears to be iodinated at the interface colloid-thyroid follicle cell [10], to produce MIT (monoiodotyrosine) and DIT (diiodotyrosine) [8]. Specific tyrosyl residues are coupled within the backbone structure of Tg, and this is the material stored in the colloid of the thyroid follicle [10], in the form of a matrix of covalently cross-linked material.

THs are stored in the colloid as part of the iodinated Tg molecule. Therefore, prior to their secretion from the thyroid gland, T_4 and T_3 must be released from the peptide linkage within Tg [10].

1.3.4 Thyroxine production and release

Thyroid hormone liberation begins with the solubilisation of Tg from its matrix of covalently cross-linked material. Proteolysis is a necessary prerequisite for solubilisation of Tg from the globules and must precede its endocytosis by thyroid epithelial cells, leading to the rapid liberation of T_4 [10].

Mature and proteolytically active cathepsins belonging to the papain family have been shown to provide thyroid epithelial cells with a mechanism of extracellular

degradation of Tg at the apical plasma membrane [21]. The proteolytic enzymes described as being involved in solubilisation of Tg and its posterior degradation are **cathepsin B**, L and K.

Friedrichs et al. [22] have shown that the lack of expression of single or multiple cathepsins caused a reduction in circulating levels of T_4 and altered the histological appearance of the colloid itself. Specifically, in the absence of enzymes (cathepsins B and L) that solubilise the cross linked Tg in the colloid, the material cannot be removed from the colloid and the follicle continues to expand as the result of continued synthesis of Tg [10].

The secretion of lysosomal enzymes from thyroid epithelial cells is a regulated process. Secretion of mature cathepsin B is triggered by TSH [21] and it is transported to endososmes/lysosomes in which it matures to become an active peptidase. After endocytosis, the Tg backbone is broken down by the action of lysosomal enzymes after fusion of the endosome with a secondary lysosome [10], as schematically presented in **figure 1.6**. The enzymes responsible for Tg degradation are not unique to the thyroid gland but are common lysosomal enzymes [10].

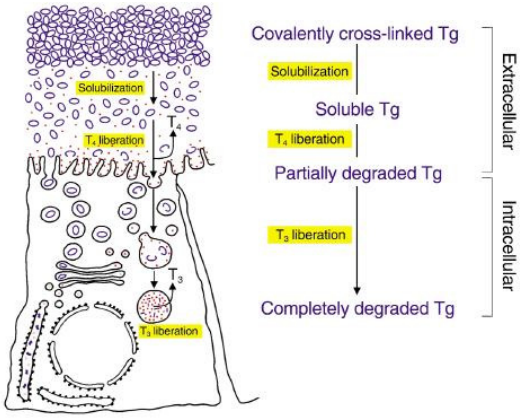

Fig 1. 6: Schematic representation of T_3 and T_4 production and liberation through Tg solubilisation and degradation by the activity of proteolytic enzymes such as Cathepsins B, L and K, in the thyroid follicular cells. Taken from [22].

The metabolic pathway required to liberate T_4 and T_3 from the Tg molecule is an important physiological event and its potential disruption by environmental chemicals may be an important mechanism by which adverse effects of specific toxicants can occur [10].

1.4 Endocrine disruptors

Endocrine disruptors (EDs) are exogenous substances that act like hormones in the endocrine system and disrupt the physiologic function of endogenous hormones [7].

Endocrine-active compounds can be natural and synthetic hormones or industrial chemicals which interfere by stimulating or inhibiting the binding or synthesis of hormones or their receptors and binding proteins [23].

EDs act in very small doses but generally over a long period of time. So, the relevant doses of EDs in the environment are minute, but with the capacity of affecting the organisms. Since the majority of chemicals with ED ability are also persistent in the environment and bioaccumulative, it's difficult to establish a direct relation to the effects of one single substance [24].

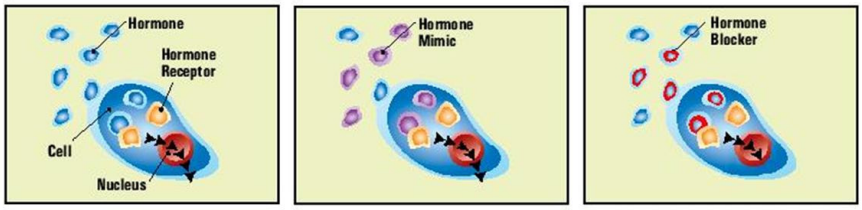

Fig 1.7: Schematic representation of the normal response of a cell to a hormone (left) and for the some of the endocrine disruption mechanisms – hormone mimic (middle) and hormone blockage (right). Image taken from [25].

Some chemicals **mimic** a natural hormone, leading the body to respond to the stimulus (as in **figure 1.7**, middle), or respond at inappropriate times. Other EDs **block** the effects of a hormone by binding to certain receptors (**figure 1.7**, bottom). In addition, others directly **stimulate or inhibit** the endocrine system and cause over or underproduction of hormones [26].

1.4.1 Thyroid disruptors – Goitrogens

The sensitive and tightly regulated feedback control system (thyroid gland autoregulation) and the large intrathyroidal storage pool of THs serve to provide a constant supply of thyroid hormone to peripheral tissues, even if perturbations are imposed by external environment, chemicals and drugs [8]. This work focused on perturbations of the HPT axis by chemicals and drugs. Many drugs affect the transport, metabolism, action and excretion of T_4 and its derivatives as well as regulation at all

levels of the HPT axis [8].

Irrespective of their mechanism of action, they are collectively called **goitrogens**, because as a result of a decrease in serum TH level, TSH secretion is enhanced, causing goiter formation. Among the goitrogens, the least toxic and those possessing the highest thyroid-inhibiting activity are clinically used in the treatment of hyperthyroidism [8].

1.4.1.1 **Anti-thyroid drugs**

An anti-thyroid drug is any agent or substance which suppresses, prevents or opposes the biosynthesis of thyroid hormones [27]. According to their principal mode of action on thyroidal iodine metabolism, antithyroid drugs are divided into two categories: 1) the monovalent anions which inhibit iodide transport into the thyroid gland, and 2) a large number of compounds that act through inhibition of thyroidal iodide binding and iodotyrosine coupling. The most important representatives of this latter category of compounds are the group of thionamides. Certain monovalent anions inhibit the transport of iodide into the thyroid gland and thereby depress iodide uptake and hormone formation [8].

On the other hand, the thionamide group of goitrogens does not prevent transport of iodide into the thyroid gland, but rather impairs covalent binding of iodide into Tg. They may be competitive substrates for TPO, preventing the peroxidation of iodide by this enzyme [8].

From a medical point of view, anti-thyroid agents can be defined as agents that are used to treat hyperthyroidism by reducing the excessive production of thyroid hormones [27] the most commonly used drug is **Propylthiouracil** (**PTU**).

1.4.1.1.1 **Propylthiouracil** (**PTU**)

PTU is a derivative of thiocarbamide. It is an anti-thyroid, reversible goitrogen drug, effective and safe in the treatment of hyperthyroidism [28] with a well-known mechanism of action: it inhibits intrathyroidal synthesis of thyroid hormones by

interfering with the iodine utilization by TPO and with the coupling of MIT and DIT required for the formation of T_3 and T_4. Furthermore, it blocks the peripheral conversion of T_4 to T_3 [29], thus maintaining the T_4 concentration in blood at a higher level that is normal.

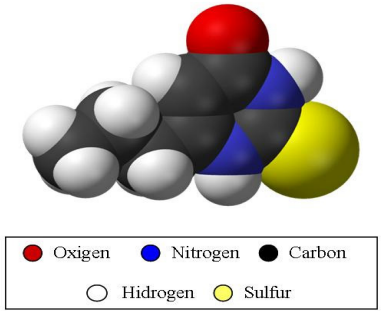

Fig 1.8: 3D Molecular structure of the thyroid disruptor PTU. Taken from [7].

According to INFARMED, Autoridade Nacional do Medicamento e Produtos de Saúde, I. P., (the Portuguese entity for medical drugs regulation) the doses for PTU for clinical application are 300 to 600 mg/day for adults until clinical control, following a gradual reduction to maintenance doses of 50 to 100 mg/day [30].

1.4.1.2 Environmental thyroid disruptors

A large number of substances may affect thyroid gland function and thyroid hormone metabolism and action. The list continues to grow with the introduction of new diagnostic agents, drugs and food additives [8].

A number of chemical contaminants are suspected to display endocrine disrupting activity at environmentally relevant concentrations and may interfere with normal growth and developmental processes that involve THs [31-33].

Environmental chemicals may interfere with thyroid homeostasis through many mechanisms of action, such as binding to transport proteins, in cellular uptake mechanisms or by modifying the metabolism of THs. Several environmental chemicals have a high degree of structural resemblance to T_4 and T_3, and therefore interfere with binding of THs to receptors or transport proteins.

There is substantial evidence that polychlorinated biphenyls (PCBs), dioxins and furans cause hypothyroidism in exposed animals and that environmentally occurring doses affect human thyroid homeostasis. Similarly, flame retardants reduce peripheral TH levels in rodents, but human studies are scarce. Studies also indicate thyroid disruptive properties of phthalates, some stimulate TH production, contrary to most other groups of chemicals [34].

The aquatic environment and organisms such as fish are constantly threatened by pollution resulting from human activity [35]. Pharmaceutics and personal care products (PCPs) represent a source of prevalent contaminants in the aquatic environment, and many can be present as complex mixtures within municipal waste effluents. One such contaminant is the bactericidal agent **triclosan** (TCS) [36].

1.4.1.2.1 Triclosan

TCS (2,4,4' – trichloro -2' - hydroxydiphenyl ether; synonym: Irgasan DP 300) is a synthetic, broad-spectrum antimicrobial agent that in recent years has exploded onto the consumer market [37]. It is used in the manufacture of a variety of commercial products including clothing, materials for food processing, PCPs (e.g. soaps and toothpaste) and surgical items (e.g. sutures) [38-44].

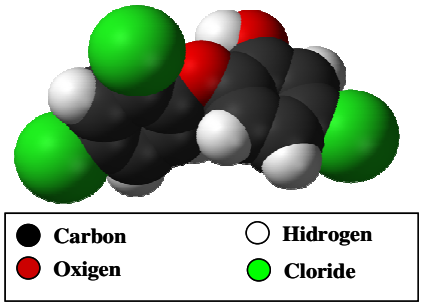

Fig 1.9: 3-D molecular structure of the thyroid disruptor Triclosan. Taken from [7].

Rather than being a general biocide (a chemical substance that disrupts so many cellular functions at once that bacteria encountering it simply cannot survive), TCS may instead be a specific biocide, killing bacteria by targeting specific cellular functions by blocking essential enzymes for fatty acid synthesis. However, this allows the bacteria to mutate, thus building up resistance and developing into "superbugs" [45].

Its ubiquitous usage in consumer products has led to widespread environmental contamination evidenced by detection of TCS in wastewater effluent in the US, UK, Japan and other countries [38-44]. Concentrations in the range of 0.01 – 0.65 μg/L in sewage effluent and 0.4 – 12 μg/L in sludge have recently been recorded from sewage treatment plant sites in North America and Europe [38-44].

During wastewater treatment, many of the chemicals, including biocides, are removed, but some chemicals still reach surface water [46]. It's the case of TCS, making this an important aquatic environmental contaminant.

The release of TCS into the environment is of particular concern as it is structurally similar to THs (**fig 1.10**) and may, therefore, represent a potential disruptor of TH action particularly as TCS or its derivatives bioaccumulate in the tissues of wildlife species [35].

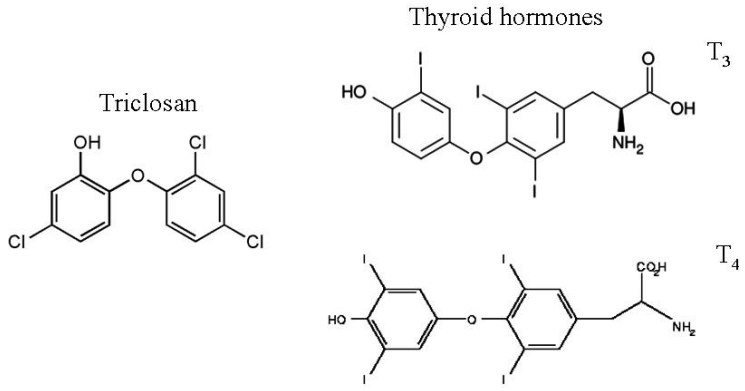

Fig 1. 10: Chemical structures of the THs (T3 and T4) and of the goitrogen TCS. Taken from [7].

1.5 Objective

The number of chemicals to which humans are exposed increases as do the quantities which accumulate in the environment, the real effects of these chemicals remain unknown but they may alter whole body homeostasis by affecting the endocrine system. Among all the glands that belong to the endocrine system, this work focuses on the HPT axis, especially the thyroid gland. There are studies which show that the HTP axis is very susceptible to some classes of man-made chemicals, especially as some of those chemicals show high structural resemblance to TH hormones, thus changing the equilibrium needed for optimal functioning of the thyroid gland. In some cases of TH

alterations, the changes may be detected as goitrogen, i.e. the enlargement of the thyroid gland. To evaluate the presence of such abnormalities in the thyroid gland, histological techniques were applied.

The thyroid gland enlargement occurs at a molecular level by alterations to TH synthesis. Among the proteins involved in TH synthesis, Tg, CtsB, TSH, NIS and TPO are among some of the most important, and are susceptible to the presence of some chemical. Using molecular tools, such as qRT-PCR, the level of expression of the mRNA for these proteins was evaluated in this study.

The goal of this work was to study the effects of TCS on the disruption of the thyroid axis by evaluating its effects through a molecular and functional analysis in an *in vivo* assay.

1.5.1 Goitrogen choice

The potential endocrine disrupting behaviour of TCS was studied in rats as it has previously been reported to alter the level of thyroid hormones in animals [47]. Moreover, the daily exposure to this agent is not insignificant as it has widespread applications in everyday products such as detergents, kitchen sponges, soaps, deodorants, cosmetics, lotion, antimicrobial creams, (…), various plastics including children's toys, paint, wallpaper, flooring, textiles, curtains, keyboards, countertops [48].

1.5.2 Experimental Model

Since the present study consisted of an *in vivo* study of TCS, it was necessary to choose a suitable animal model. Having in mind that the objective of the work included the functional analysis of the goitrogenic action of TCS, the **zebrafish** was chosen.

Zebrafish (*Danio rerio* a small tropical freshwater fish) from the family Cyprinidae that rarely grows beyond 50–60 mm in length and was first used as a genetic model system is the early 1980s. The zebrafish shares numerous anatomical similarities with higher vertebrates, including humans, both in the general body plan and in specific

organs. Close parallels exist in many aspects of early embryogenesis and in the anatomical and histological features of the brain, spinal cord, sensory systems, cardiovascular system, and other organs. Not infrequently, genetic defects in zebrafish resemble human disorders [49].

Also the large base of established knowledge on the developmental biology and genetics of the zebrafish and the detailed genetic map of this species genome facilitates the identification of modifications in gene expression during endocrine disruption [50].

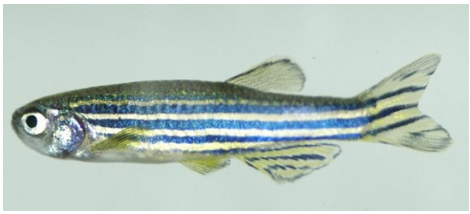

Fig 1.11: *Danio rerio* (zebrafish).

2. Methodology

The experimental work consisted of *in vivo* exposure of zebrafish to a goitrogen (TCS) in order to evaluate its effects on thyroid gland morphology and mRNA (messenger ribonucleic acid) expression of key genes encoding proteins involved in the synthesis of the THs namely, TSH in the pituitary gland and Tg, TPO, NIS and isoform a of CtsB in the thyroid.

The fish were exposed orally to PTU a goitrogen commonly used in hyperthyroidism treatment and whose effects on thyroid hormone levels are well studied for different classes of organisms, including fish [23, 51] and the bactericide TCS. The results of administering PTU served as a reference to which the effects of TCS could be compared.

For morphological evaluation of the thyroid, the whole head was processed for histological analysis and several parameters were measured in thyroid follicles, such as internal and external areas, vacuolation and height of the epithelium.

The evaluation of whole head mRNA expression was achieved through the application of a number of molecular tools, such as Polymerase Chain Reaction (PCR), for amplification and cloning of templates and qualitative analysis of tissue expression for key genes, and real-time quantitative PCR (qPCR) to analyse modifications in gene expression. For the successful application of the previous tools, *in silico* identification of the target gene was needed, to obtain the predicted cDNA sequences of the proteins of interest.

2.1 Experimental conditions

For the duration of the treatment, 21 days, a total of 48 zebrafish imported from Thailand, were weighted and randomly distributed into 6 tanks (8 individuals per tank), containing 5,5 L of freshwater at room temperature (26ºC) and under 12h light:12h dark photoperiod. Water aeration and circulation were achieved using a pump, and water was renewed once a week by replacing approximately 2/3 of the total volume of the tank

with dechlorinated fresh water. The fish were fed once a day with dry food pellets at 3% per kg of fish.

2.1.1 Goitrogen dose

The treatment with TCS (or Irgasan, Fluka, Sigma-Aldrich, Madrid, Spain) was carried out at a daily dose of 100 mg/kg. This dose was defined based on a study by Crofton *et al.* 2008 [47] in mice, which show that serum T_4 decreased in a dose dependent manner, as the dose of TCS increases. According to the results of the study, the lowest TCS concentration tested which caused a significant decrease in the T4 levels was 100 mg/Kg/day.

Treatment with PTU (Sigma-Aldrich), the positive control treatment, was carried out at a daily exposure rate of 5 mg/kg. This dose was selected based upon the information available from INFARMED [30], in which the recommended exposure, for adults was of 300-600 mg/day (assuming that a human adult weighs approximately 60 kg, the daily exposure dose consisted of 5 to 10 mg/kg/day). The dose of PTU chosen was further supported by the study by Villar *et al*, in which a PTU dose of 4.4 mg/kg/day up to 35 mg/kg/day resulted in lower concentrations of circulating T_4 [52]. Also in previous works from the Molecular and Comparative Endocrinology (MCE) group in fish, concentration of 1mg/kg/day PTU was tested. This concentration did not induced hypothyroidism [53].

2.1.2 Drug administration

The fish exposure to the goitrogens TCS and PTU was achieved orally by applying the drugs in the food. The **daily dose of food** administered to the animals in each tank was determined assuming that each fish ate approximately 3% of its total body weight. The **total mass of goitrogen** administered was determined taking into account the sum of the body weight of all the fish per treatment group, prior to acclimatization (**table 2.1**), and the previous established doses (100 mg/kg/day for TCS and 5 mg/kg/day for PTU).

Table 2.1: Total body weight (g) for the different groups (n=10) acquired prior to acclimatization.

Tank	Total body weight (g)
Control 1	3.27
Control 2	3.14
PTU 1	2.69
PTU 2	3.37
TCS 1	2.67
TCS 2	3.25

Stock solutions of TCS (1 mg/mL) and PTU (10 mg/mL) were prepared by dissolving the drugs, weighted using a precision balance, in 95% ethanol in glass tubes. The appropriate volume of stock solution required to attain the desired concentration was diluted to reach a final volume of 100 µL. The food pellets were then immersed in each drug solution (TCS and PTU) or in ethanol only (control group) and then allowed to dry in a fume cupboard.

The dried food pellets were administrated to replicate tanks (n=2) for each treatment.

2.2 Sampling

After 21 days treatment, all the animals were collected (n = 8 per tank) and placed in a vessel containing anaesthetic (2-phenoxyethanol, Sigma, diluted at 1:2,000 in fresh water) in order to euthanize the animals. Once euthanized, the animals were weighted and their standard length [54] measured. Half of the individuals from each tank were immersed in liquid N_2 and stored at -80ºC until RNA extraction, while the other half were immersed in fresh 4% paraformaldehyde (PFA[1]) after nicking the abdomen to ensure good penetration of the fixative and stored at 4ºC.

2.3 Histological Analysis

The histological analysis was carried out on the tissues of the whole head, where the thyroid gland is located, due to the small size of the animals and the dispersed nature

[1] ***Vide*** **Annex For 4% PFA preparation**

of the thyroid tissue in fish. The animals (8 fish per group) immersed in the 4% PFA fixative were washed twice in PBS 1X, 30 minutes each, and a final wash with diethylpyrocarbonate (DEPC)-treated water[2] carried out for 30 minutes. The samples were then immersed in ethanol 70% in DEPC-treated water and stored at 4ºC.

2.3.1 Tissue processing

The head was isolated from the rest of the animal by cutting off the operculum (gill cover) followed by severing of the head. All tissues were decalcified (removal of calcium ions to soften tissue) [7] in EDTA 0,5M, pH 8,0 for 6 days with agitation and kept away from light.

Decalcification of the head tissues was followed by paraffin embedding using a Leica TP1020 tissue processor, where the tissues were dehydrated through immersion in a gradient of ethanol, 70% (10 min), 95% (30 min x2) and 100% (1 hour x2) followed by a mixture of ethanol:xylene (1:1) (1 hour), saturated xylene (100%) (1 and 1.5 hours), a mixture of xylene:paraffin (1:1) (2 hours) and paraffin (100%) (2 hours). Once the paraffin embedding ended, the tissues were used to prepare the paraffin blocks in 3 consoles: thermal, dispensing and cryo (Miles Scientific), obtaining a total of 6 blocks, 4 heads per block. Serial sections of 5µm were obtained from each block using a Leica RM 2135 microtome and were mounted on glass slides coated with Aminopropyltriethoxysilane (APES)[3] (Sigma-Aldrich) and dried overnight (ON) at 37 ºC.

2.3.2 Staining

In order to identify the thyroid structures, the tissues were stained using the Cleveland-Wolfe trichrome method. This method stains the colloid in thyroid follicles bright red while the epithelium stains blue or purple.

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[2] ***Vide*** **Annex For DEPC treated water**

[3] ***Vide*** **Annex for APES slide coating procedure**

The first steps of this staining consist of removal of paraffin wax and rehydration of the tissues. The tissues were immersed for 15 min in K-Clear (Kaltec, Padova, Italy), twice, followed by the immersion in a descending series of ethanol dilutions (100% for 10 min, 95% and 70% for 5 min each). The rehydration ended with a 5 min immersion in distilled water. Rehydrated tissues were immersed for 5 min in Erlich's haematoxylin followed by a wash in tap and distilled waters. The tissues were dipped for 5 min in 1% aqueous Erythrosine and washed with tap and distilled waters. The tissues were stained for 8-10 sec with Orange G (2% in 1% phosphotungstic acid) followed by a fast wash in distilled water before staining with Aniline Blue (0,25% aqueous solution acidified to pH 3-4) for 90 sec. The tissues were then quickly washed in 100% ethanol, incubated 2 x 5 min in K-Clear and mounted for definitive preparation in DPX (Fluka, Sigma).

The tissues were examined in an Olympus BH2 microscope for the presence or absence of colloid and vacuolation in thyroid follicles, follicular epithelial folding and evaluation of follicular size. The internal/external areas and the cell height were measured for sections from control (n = 4), TCS (n = 6) and PTU (n = 4) treated zebrafish using the software ImageJ (freeware-NIH) [55] for digital analysis from the photographs taken from an Olympus DP11 digital camera. The cell height of 2 different thyrocytes per follicle, lying 90º from one another, was also measured for 4 individuals in each of the experimental groups.

2.4 Molecular Analyses

To study if the goitrogens had an effect in the level of expression of the mRNAs for key proteins involved in TH synthesis (CtsBa, Tg, TPO, TSH and NIS), it was important first to isolate the genetic material – the RNA from whole head tissues (where the thyroid gland and pituitary gland are found). So, prior to RNA extraction, the head and body were separated.

As the study was centred on specific gene products, it was also important to test their presence in the extracted RNA. This was achieved by complementary DNA (cDNA) synthesis from whole head RNA from control fish, and the use of specific primers for PCR. These PCR reactions also provided the specific cDNAs to clone each

gene product into a vector, and each cDNA fragment + vector construct was introduced into host cells transforming them. After multiplication of the host cells, each construct was extracted, its identity was confirmed by digestion and sequencing, and it was also used as a standard in qPCR with specific primers, which allowed the quantification of gene expression in the different experimental groups.

2.4.1 RNA extraction and Quantification

For RNA isolation from the head, frozen animals (n=8 per group) were placed on a hard surface, and the heads were severed with a sharp blade. To avoid RNA degradation by the RNases present in the tissues, they were maintained in liquid N_2 for part of the tissue isolation.

The extraction was performed using TRI reagent (TRI, Sigma-Aldrich), following the protocol provided by the company and a manual glass homogenizer. The previously severed and crushed tissue was removed from liquid N_2, immersed in 400 µl of TRI at room temperature and homogenized. The final mixture was transferred to a fresh tube and incubated at room temperature for 5 minutes. 0.2 mL of chloroform/ml TRI was added; the mixture was vigorously mixed and left to incubate at room temperature, and then centrifuged at 12000xg, 15 min, 4ºC. The (superior) aqueous phase was carefully removed to a fresh tube to which 0.5 ml isopropanol/ml TRI was added. The solution was incubated at room temperature followed by a centrifugation step at 12000xg, 15 min, 4ºC to pellet the RNA. The isopropanol was discarded and the pellet washed twice with 1 ml of cold ethanol 75% in DEPC-treated water/ml TRI used. The mix was centrifuged at 12000xg, 10 min, 4ºC and ethanol removed by inversion of the tube. The pellet of RNA was allowed to dry on ice and ressuspended in 200 µl DEPC-treated water (to avoid RNase contamination). 9 µl of ressuspended RNA (diluted 1:10) was applied on 1.5% electrophoresis to evaluate RNA integrity, another aliquot was stored directly at -80ºC (working aliquot), while 2.5 V (volumes) of 100% ethanol and 0.1 V of 3M potassium acetate (KAc) were added to the remaining volume and stored at -80ºC (long-term storage aliquot).

The RNA in the samples was quantified using the Qubit™ fluorometer and the Quant-iT™ RNA Assay kit (Invitrogen, Carlsbad, CA, USA). Quant-iT™ Assay kits utilize advanced fluorophores that become fluorescent upon binding to DNA, RNA, or proteins; the fluorescence intensity of the resulting complex depends directly on the amount of the target molecule in the sample. The Quant-iT™ Reagents only report the molecule of interest – there's no interference from free nucleotides or other contaminants (the assay Quant-iT RNA assay is highly selective for RNA over double-stranded DNA)[56].

As described in the protocol provided with the kit, the Quant-iT™ working solution was obtained by diluting of Quant-iT™ RNA reagent 1:200 in Quant-iT™ RNA buffer. 1 µl of the samples (each RNA diluted 1:10) and 10 µl of each standard (standard 1 and 2) were loaded in the assay tubes to which 199 µl and 190 µl of working solution were added to the tubes, respectively. The equipment was calibrated with the prepared standards followed by the reading of the samples concentrations.

2.4.2. Removal of genomic DNA contamination

During the RNA extraction, it is isolated from a mixture of DNA and other molecules such as proteins and lipids. Although a great part of the genomic DNA (gDNA) is removed during extraction with TRI reagent, it is necessary to assure that all the gDNA contamination is removed prior to qRT-PCR and for this reason samples were treated with DNase. This step was carried out using the DNA-free™ Kit (Ambion®, UK).

DNase treatment was carried out on 4 µg of RNA which was transferred into a fresh tube to which 2 µl DNase buffer, 1 µl recombinant DNase I, and nuclease-free water, were added, to obtain 20 µl of reaction solution. The solution was mixed gently and incubated for 30 min at 37ºC. 2 µl of inactivation reagent were added and incubated at room temperature for 2 min with occasional vortexing. The mixture was centrifuge at 10000xg, 1.5 min, 4ºC, placed on ice and the supernatant was carefully transferred to a fresh tube. 1 µl of a 1:5 dilution of the treated solution was quantified as described in section 2.4.2.1.

2.4.3 Complementary DNA (cDNA) synthesis

cDNA is a single-stranded DNA molecule with a nucleotide sequence that is complementary to an RNA molecule, and it is synthesised by the enzyme reverse transcriptase (RT) from an RNA template [57]. It also differs from genomic DNA because, like mRNA, it does not contain non-coding sequences that are present in genes, the introns.

The synthesis of cDNA (reverse transcription) was carried out in a Robot-Cycler thermocycler (Stratagene, La Jolla, USA) using 500 ng of DNAse-treated RNA, 200 ng of $pd(N)_6$ random hexamers (GE Healthcare, UK), 1 µl of deoxynucleotide-triphosphates (dNTPs) (GE Heathcare, 10 mM each) and sterile water to a final volume of 13µl. The mix was heated to 65ºC for 5 min and incubated on ice for 5 min. 40U of MMLV reverse transcriptase (Promega, VWR, Portugal), 5U of RNAguard Rnase inhibitor (GE Healthcare) and 1x reverse transcriptase buffer (Promega) were then added, in a final volume of 20µl. The samples were run in a synthesis reaction for 10 min at 25ºC, 50 min at 42ºC synthesis was terminated by incubation for 5 min at 72ºC. A –RT control (cDNA synthesis in the absence of RT enzyme) for one of the samples was also performed, to control for genomic DNA contamination.

2.4.4 *In silico* identification of predicted cDNA sequences

The *in silico* studies allowed the identification of the gene sequences that correspond to the gene encoding the proteins to be studied as well as acquiring information about the cDNA sequences.

Predicted cDNA nucleotide sequences for the genes of interest (CtsBa, Tg, TPO, TSH and NIS) were obtained by searching the zebra fish genome database of the National Centre for Biotechnology Information (NCBI, www.ncbi.nlm.nih.gov), using the query by gene name. The predicted cDNAs were retrieved from the database, and their identity was confirmed by comparison of their translated sequence with homologous proteins from other organisms, using BlastX against the non-redundant protein sequence database (nr) [58].

For the target genes that were not found in the genome using the query by gene name, homologous cDNAs from other organisms were retrieved from NCBI and used to find the predicted protein in the zebra fish genome by BlastX at the NCBI site. The predicted zebra fish cDNA was retrieved and its identity confirmed by BlastX against the nr protein database.

In order to find the cDNA/protein sequence for genes with high homology to the target genes, which could be co-amplified by PCR due to cross-reaction, the predicted cDNAs of the target genes were blasted against the zebra fish genome (BlastX). Whenever proteins with high homology were found, the corresponding cDNA nucleotide sequence was retrieved and aligned with the cDNA of the target gene using ClustalX 2.0.9 software. These alignments were further used for specific primer design used in the following PCR techniques.

2.4.5 Polymerase Chain Reaction (PCR)

PCR is a process that specifically generates large amounts of DNA *in vitro*, starting from a small amount of a DNA template, with a three-step cycling process. For this amplification to occur the essential components for PCR amplification are (1) two synthetic oligonucleotide **primers**, complementary to regions on opposite strands that flank the target DNA sequence, and that, after annealing to the template DNA, have their 3' hydroxyl ends oriented toward each other; (2) a **target sequence** in a DNA sample that lies between the pair of primers; (3) a **thermostable DNA polymerase** that can withstand multiple cycles of heating to 95ºC or higher; and (4) the four **dNTPs** (adenine, tyrosine, guanidine and cytosine).

The first step in the PCR amplification system is the thermal **denaturation** of the DNA sample by raising the temperature to 95ºC. In addition to the template DNA this reaction contains a vast molar excess of the two primers. For the second step, **renaturation** or **annealing**, the temperature of the mixture is slowly cooled to ≈55ºC (depending on the primer sequences). During this step, the primers base pair with their complementary sequences in the template DNA. In the third step, **synthesis** or **extension**, the temperature is raised to ≈72ºC, which is optimum for the catalytic functioning of *Taq* DNA polymerase in extending the primers to make copies of the DNA template [59]. These three steps are repeated for a number of cycles (usually 20-

35) and the amount of target DNA duplicates in each cycle, allowing the exponential amplification of the amount of specific DNA in the reaction mixture.

2.4.5.1 18S ribosomal (r)RNA PCR

To assess the quality, reliability of the synthesised cDNA (section 2.4.3), the step of synthesis was followed by PCR amplification of the gene for the 18S ribosomal RNA (18S), which served as an internal control, as it is expressed in abundance in all organisms and cell types.

The reaction mix, for each sample for the 18S PCR was composed of 2.5 µl of 10x reaction buffer, 0.75 µl of 50 µM $MgCl_2$, 0.1 µl of Taq DNA polymerase (5U/µL,EuroTaq, EuroClone® Genomics, UK), 18.35 µl of milliQ water, 0.3 µl of dNTPs 10mM each, forward and reverse primers (10 pmol/µl) 1 µl each, and 1 µl of the synthesised cDNA (diluted 1:10).

The primer sequences are indicated in **table 2.2**:

Table 2.2: Primer sequences for 18S PCR reactions, forward (Fw) and reverse (Rw), previously designed by a member of the Molecular and Comparative Endocrinology (MCE) group.

Primer name	Sequence (5' → 3')
18S forward	TCAAGAACGAAAGTCGGAGG
18S reverse	GGACATCTAAGGGCATCACA

The 18S PCR reaction was carried out in the i-Cycler thermocycler (Bio-Rad) and initiated with a denaturation step at 95ºC, 2 min, followed by 25 cycles of denaturation (95ºC, 45 seconds), annealing (59ºC, 30 seconds) and synthesis (72ºC, 45 seconds). A final elongation step was performed at 72 ºC, 1 min.

PCR products (5 µl) were applied on a 1.5% agarose gel and analysed by electrophoresis.

2.4.5.2 Primer design for target gene cloning

The specificity of the PCR reactions targeting the genes encoding for Tg, TPO, TSH, NIS and CtsBa was determined by the design of specific primers for each gene, which assured that the amplified sequence matched the gene to be studied.

The design of the specific primers for the predicted cDNA sequences acquired in section 2.4.4 was performed using the software Primer Premier™ (Biosoft International) choosing the primers with less or most instable secondary structures (such as dimers or hairpins), low probability of false priming and targeting cDNA in regions with low homology to related gene sequences, identified by carrying out sequence alignment.

Based upon the preceding principles the primers presented on **table 2.3** were selected.

Table 2.3: cDNA accession numbers acquired from the *in silico* studies, primer sequences for RT-PCR reactions, forward (Fw) and reverse (Rw), complementary to the cDNA sequences of the genes encoding for the proteins Tg, CtsBa, TPO, NIS, TSH and GAPDH-2. The amplicon (PCR product) length is indicated in base pairs (bp).

Gene	cDNA Accession number		Primers Sequence (5' → 3')	Product length (bp)
Tg	DQ278875.1	Fw	TCACAGCATCAATGCTGCG	771
		Rv	TGGAGGTTTTTGCGGTTCA	
CtsBa	BC056688.1	Fw	CTTGGACGGCTGGACATAAC	922
		Rv	TATAACATTTGATCAGGGGCTTC	
TPO	XM_692744.3	Fw	AACCCAAAGGCTGGAACGCTG	890
		Rv	AGAGATGGTGACATGCCCGAAG	
NIS	DQ402039.1	Fw	GAATGAGGTTTGGCAGAGGG	618
		Rv	GGTACGGCATGTACTGGTCAGG	
TSH	AY135147.1	Fw	AGACCCTCCAGACAGACATCC	459
		Rv	GCGTAGTTGTTCTCCTCGG	
GAPDH-2	AY818346	Fw	CTTTGGTATTGAGGAGGC	533
		Rv	GGAATGGTCTGGCTTTTCT	

The optimization of the PCR reaction conditions was then carried out, by choosing the optimal annealing temperature (Ta) and $MgCl_2$ concentration for each pair of primers.

2.4.5.3 Optimization of PCR conditions

The choice of Ta is largely determined by the melting temperature (Tm) of the two PCR primers [60], estimated by the software. Since the behaviour of new primers during a PCR reaction is not well known, a gradient of temperatures was tested. For the concentration of $MgCl_2$ needed in the reaction, highly stringent conditions, with $[MgCl_2]$ =1mM, to medium and low stringent conditions, with $[MgCl_2]$ =2-2,5mM, were tested, combined with the temperature gradients.

After optimization, the final PCR conditions for amplification of the target genes were identified and are presented in **table 2.4.** The PCR conditions selected allowed the reaction product to be visualised on an agarose gel and confirmed that the PCR product had the expected size and there was only minimal contamination with other reaction products (indicated by the presence of additional bands).

Table 2.4: PCR conditions for the reaction mix and cycling conditions used in the PCR of the different target genes. For the reaction mix the values are the volumes in µL for one reaction of 25µL. The DNA template was 2µL of cDNA from control fish or 5µL of a PCR product previously obtained from 2µl of cDNA using the same PCR conditions (re-amplification, samples marked with *).
The optimized annealing temperatures (Ta) and number of cycles are shown in the top row of the table. The duration (in minutes –' – or seconds – '') of each PCR step ([a] – first step of denaturation; [b]- steps of cycle repetitions - denaturation, annealing and synthesis; [c] – elongation step) are also indicated in the "PCR steps" panel.

		Tg (T_a = 60,4ºC) PCR cycles 30	CtsBa (T_a = 59,3ºC) PCR cycles 30	TPO (T_a = 59,9ºC) PCR cycles 40	NIS (T_a = 52,0ºC) PCR cycles 30	TSH (T_a= 63,4ºC) PCR cycles 30	GAPDH-2 (T_a= 60,0ºC) PCR cycles 40
Reaction mix	Reaction Buffer (160 mM)	2,5	2,5	2,5	2,5	2,5	2,5
	MgCl2 (50 mM)	1,25	1	1	1	1	0,75
	dNTPs (10 mM)	0,5	0,5	0,5	0,5	0,5	0,5
	Primer Fw (10 mM)	1	1	1	1	1	1
	Primer Rv (10 mM)	1	1	1	1	1	1
	water milliQ st	13,9	13,9	16,9	13,9	13,9	18,15
	cDNA/PCR product	5*	5*	2	5*	5*	1
	Taq DNA Polymerase	0,1	0,1	0,1	0,1	0,1	0,1
PCR steps	95ºC[a]	2'	2'	2'	2'	2'	2'
	95ºC[b]	30''	30''	30''	30''	30''	45''
	Ta[b]	48''	1'15''	45''	45''	45''	30''
	72ºC[b]	20''	20''	30''	20''	20''	45''
	72ºC[c]	7'	7'	5'	7'	7'	7'

The PCR reaction products were analysed by 1.5% agarose gel electrophoresis (5µl) and the size of the amplified bands compared with that predicted from the cDNA sequence. For some genes where no visible PCR product was found on the gel, a re-amplification was carried out using the same PCR conditions and 5µL of the PCR

product as template (Table 2.3). Positive PCR products, containing only the reaction product of the expected size were cloned into a plasmid cloning vector.

2.4.6 Cloning and transformation

Molecular cloning is a process in which a fragment of DNA is inserted in a cloning vector (e.g. a plasmid) for subsequent production of many DNA copies (propagation) in a host which is usually bacteria [61].

A plasmid cloning vector is a plasmid which possesses determinant features, such as (1) a small size, which is necessary because the efficiencies of transfer of exogenous DNA into *Escherichia coli* (*E.coli*) decreases significantly with plasmids that are more than 15kb long; (2) a choice of unique restriction endonuclease recognition sites into which the insert DNA can be cloned (multiple cloning site); (3) one or more selectable genetic markers for identifying recipient cells that carry the cloning vector-insert DNA construct [59].

The recombinant DNA molecules are then introduced into host cells, where they replicate, producing large numbers of recombinant DNA molecules that include the fragment of DNA originally linked to the vector [61]. The introduction of the construct (DNA insert+vector) into the host cells is called transformation. After transformation, the exogenous DNA in the host cells can be extracted to be used in future studies.

2.4.6.1 Cloning

In the present study the cloning vector chosen was the plasmid pGEM®-T Easy[4] (Promega). This plasmid allows the direct cloning of PCR products with 3'- deoxyadenosine (A) residues at the extremities as a results of the 5´_3´ exonuclease activity of some DNA polymerases, including Taq polymerase. The ends of the PCR product are complementary to the 3'- thymidine (T) extremities present at the insertion site of the plasmid and greatly improves the efficiency of ligation of the PCR product to the plasmid cloning site by the enzyme T4 DNA ligase. The pGEM-T plasmid has its

[4] *Vide* Annex For plasmid vector map and reference points

multiple cloning site within the α-peptide coding region of the enzyme β-galactosidase. This enzyme is responsible for X-Gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside) degradation, producing blue coloured colonies of bacteria containing the plasmid. Insertional inactivation of the α-peptide allows recombinant bacterial clones containing plasmid with an insert to be directly identified by colour on a selective plate. The ligation reaction was composed of the reagents and quantities presented on **table 2.5**.

Table 2.5: Reaction components for the ligation of the PCR products of the several genes to the plasmid pGEM-T Easy (indicated in volume in µL, in a reaction with 10µL in total).

		Tg	CtsBa	TPO	NIS	TSH	GAPDH-2
Reaction Components (µL)	DNA	4,2	8	8	1,5	1,5	4,2
	Enzyme buffer (2x) (10x for CtsBa and TPO)	5	1	1	5	5	5
	pGEM-T Easy	0,3	0,3	0,3	0,3	0,3	0,3
	T4 DNA ligase	0,5	0,5	0,5	0,5	0,5	0,5
	Water st	-	0,2	0,2	2,7	2,7	-

The mixture of the ligation reaction was incubated ON at 4ºC and the product used in the transformation of competent cells.

2.4.6.2 Transformation

The bacterial cells used in the study were the strain *E. coli* XL-1 Blue MRF' (Stratagene), treated and aliquoted previously by a member of the Molecular and Comparative Endocrinology (MCE) group for competence by the calcium chloride method[5]. Competent cells are those that possess more easily altered cell walls and DNA can more readily enter into the bacteria, so these cells readily incorporate foreign DNA.

For bacterial transformation, 4 µl of the ligation mix were added to 100 µL of competent XL-1 Blue MRF' cells, and incubated for 30 min on ice. The cells were subjected to a thermal shock, 2 min at 42ºC and 2 min on ice, and plated on solid LB

[5] Vide Annex for calcium chloride competence method protocol

selective medium[6], with 75µg of ampicilin/mL, 80 µg/ml X-Gal and 0.5 mM isopropyl-beta-D-thiogalactopyranoside (IPTG), and allowed to grow ON at 37ºC.

After the incubation the plates were analysed and the number of white colonies (with the insert) and blue colonies (without insert) evaluated.

2.4.6.3 Plasmid DNA extraction (Minipreps)

For plasmid DNA (pDNA) extraction from recombinant bacteria the method of alkaline lysis was applied. In this method the addition of an alkaline solution (sodium hydroxide, NaOH) and a detergent (sodium dodecyl sulphate, SDS), disrupts the cell wall, dissolves the cell membrane, thus releasing the genetic material of interest to the solution.

To obtain the purified pDNA, some white colonies from section 2.4.6.2 were transferred and allowed to grow in approximately 2 mL of LB Broth with 100 µg/ml ampicilin, ON at 37ºC with agitation.

1.5 ml of culture were transferred into a fresh tube and centrifuged in a microcentrifuge at room temperature and maximum speed for 5 min. The supernatant was discarded and the pellet resuspended with 100 µl of cold GTE 1x, with RNase[7] (final concentration: 50µg/ml). 200 µl NaOH 0.2 M/SDS 1%[8] were added and mixed by inversion and kept on ice until clarified. The solution was neutralized by adding 150 µl of cold potassium acetate (KAc)[9] 3M, pH 4.8 followed by mixing until flocculation. The tubes were incubated on ice for 15 min, and centrifuged maximum speed for 15 min at 4ºC. The supernatant was collected into a fresh tube, 1 volume of cold isopropanol 100% was added and incubated at -20ºC for 20 min, following centrifugation at maximum speed for 20 min, 4ºC. The supernatant was discarded and the DNA pellet washed twice with cold ethanol 75%. The sample was left to dry and resuspended in milliQ water.

[6] *Vide* Annex For LB medium preparation
[7] *Vide* Annex for GTE 1x with RNase solution preparation
[8] *Vide* Annex for NAOH 0.2M/SDS 1% solution preparation
[9] *Vide* Annex for KAc 3M, pH 4.8 solution preparation

The purified and resuspended pDNA was digested with restriction enzymes to liberate the insert and confirm if the plasmid was successfully ligated with a PCR fragment of the expected size. The enzyme used was chosen according to the restriction sites of the plasmid[10] and the insert sequence, so that the insert would not be cleaved. The enzymatic reaction mix utilised is described in **table 2.6**:

Table 2.6: Reagents for the enzymatic digestion of pDNA (insert isolation) containing each target gene indicated in µl per digestion (*Eco*RI and *Sal*I from Promega).

	Tg	CtsBa	TPO	NIS	TSH	GAPDH-2
pDNA	4	4	1	4	7	1
OPA buffer[11]	2	2	2	2	2	2
Enzyme	*Eco*R I	*Eco*R I	*Eco*R I	*Eco*R I	*Eco*R I	*Sal*I
Enzyme volume (µL)	0.5	0.5	0.5	0.5	0.5	0.5
sterile water	3.5	3.5	6.5	3.5	0.5	6.5

The reaction was incubated at 37ºC for 1,5 hours, and digestion products were visualized by (1%) agarose gel electrophoresis.

The minipreps (pDNA) containing the DNA fragment with the expected size for each target gene were sequenced at CCMar sequencing facilities using T7 and SP6 primers, to confirm their identity.

2.4.7 Real-Time PCR

Real-time reverse transcription quantitative PCR (or qPCR) is an established technique for gene-expression analysis. Benefits of this procedure include its sensitivity, large dynamic range as well as accurate quantification of specific gene expression [62].

The assay relies on the detection of fluorescent marker which binds to the DNA [63] and the measurement of the increase in the fluorescent signal, which is proportional to the amount of DNA produced during each PCR cycle [64], which increases in an exponential like manner during the course of the reaction **(fig 2.1)**.

[10] *Vide* Annex For plasmid vector map and reference points
[11] *Vide* Annex for OPA buffer preparation

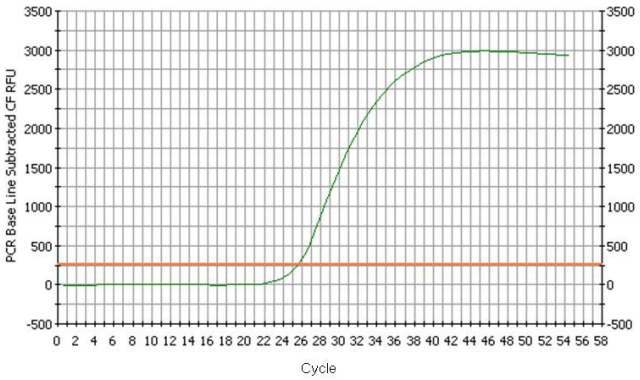


Fig 2.1: The fluorescence signal increase throughout the PCR cycles due to the DNA amplification.

Two main types of detection chemistry are most used: **TaqMan® probes** and the **SYBR Green dye**. The latter system was used for qPCR in the study. When TaqMan probes are used, primers and probes specific for the sequence of interest are added into the reaction mixture. This probe is marked with a fluorophore, which does not emit any fluorescence while it is attached. As the extension steps occur, the probe is broken down, the fluorophore released and it starts to emit fluorescence **(Fig 2.2)**. So, only specific products to which the probe binds are detected after amplification.

The SYBR Green is not as specific as TaqMan as it emits fluorescence when it binds to any double stranded DNA present in the reaction **(Fig 2.2)**. Nevertheless, it does not require the purchase of expensive fluorescent probes for each gene (adequate when we want to quantify several genes in a small number of samples) and it allows characterisation of non-specific amplification by melting curve analysis.

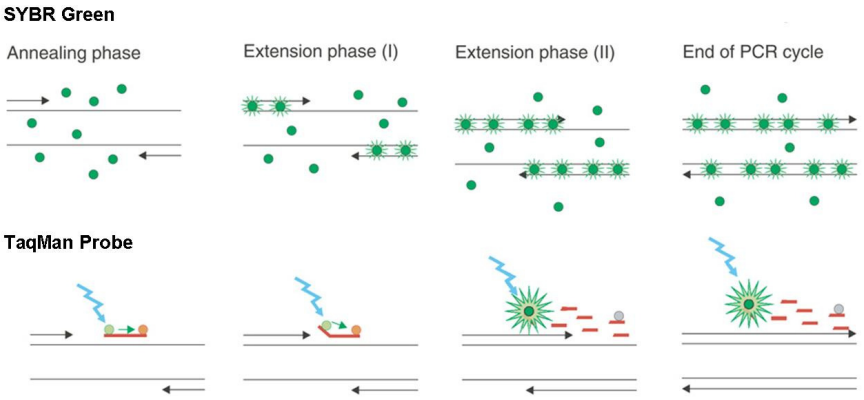


Fig 2.2: Schematic representation of the functioning of the SYBR Green dye and TaqMan probe. **SYBR Green** binds to the dsDNA as it is being synthesised, and starts to emit fluorescence. **The TaqMan probe** binds to the DNA with the primers and as the fluorophore (green) is excited, it transfers the energy to a quencher molecule (orange) present in the probe; as the extension occurs, the probe is broken down, the fluorophore is released emitting fluorescence. Taken from [65].

Individual reactions are characterized by the PCR cycle at which fluorescence first rises above a **threshold** background fluorescence (set by the user or auto-calculated by the software), a parameter known as the **threshold cycle** ($\mathbf{C_T}$). The more target there is in the starting material, the lower the C_T **(fig. 2.3)**. This correlation between fluorescence and amount of amplified product permits accurate quantification over a wide dynamic range, while retaining the specificity of conventional end-point PCR assays, but with a greatly increased sensitivity [64].

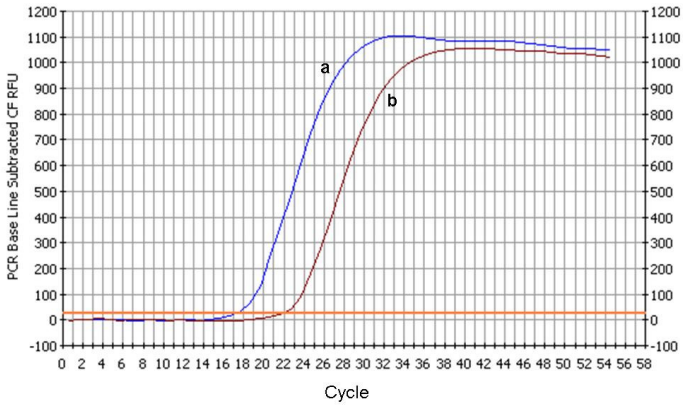


Fig 2.3: Comparison of fluorescence between two amplification plots for the same amplicon, with C_Ta = 17 and C_Tb = 22. The difference between the samples C_T is due to the fact that sample b has less starting material (sample b = 1:10 sample a). The horizontal line (orange) is the threshold of the reaction.

A standard curve is obtained by running qPCR reactions of a dilution series of a template containing the fragment of interest, such as a linearised miniprep or cDNA. This standard curve can be used to relate the quantity of amplicon amplified from a known concentration of starting material to a given C_T, which is a linear relation over a great magnitude of starting quantity. To determine the starting amount of the target gene in the cDNA samples analysed, their C_T obtained by qPCR is related to a concentration value obtained from the standard curve which is run in the same plate as the cDNAs.

Normalising to a reference gene is a simple and popular method for internally controlling for error in qPCR [62], such as the amount of starting material and variation of reverse transcription efficiencies [66].

A vast number of reference genes have been proposed for gene expression analysis. Among the most common used housekeeping genes are β-actin, glyceraldehyde-3-phophate dehydrogenase (GAPDH) and 18S rRNA, as they are expressed constitutively and are involved in basic housekeeping functions required for cell maintenance [67], and their expression is assumed to be unaffected by most experimental parameters [68]. However, it is now generally accepted that there is no

universal reference genes and that the reference genes (ideally more than one) must be validated for particular experimental conditions [69]. The candidate genes evaluated were 18S and the mRNA for GAPDH testis-specific (GAPDH-2), which are commonly used as internal reference genes in qPCR [62].

For the application of the qPCR technique to the present study to evaluate the level of target gene expression, the preparation process was very similar to that described for the PCR technique and included the design of specific primers for each gene and the optimization of reaction conditions (in this case temperature).

2.4.7.1 Primer design

Novel gene specific primers were designed with the software Beacon Designer (PREMIER, Biosoft International). The sequences analysed for the design of qPCR primers were those identified *in silico* (section 2.4.4) and limited by the primers used to obtain the cloned fragments (section 2.4.5.2).

Other factors taken into consideration in the design of these primers were:

- avoid runs of 4 of more bases, as it would reduce annealing efficiency;
- include some G/C clamps in the 3' extremity, as it facilitates the binding of the primer to the template;
- keep G/C percentage in the primers around 40 to 60%;
- avoid complementary sequences between the primers, to reduce the occurrence of secondary sequences such as hairpins, self dimers and cross-dimers;
- keep the amplicon size short (75-150 bp);
- design forward and reverse primers in a different exon or one of the primers, to the junction of two exons, so that amplification from gDNA (if there is contamination) is reduced or distinguishable by size from cDNA amplification.
- Design primers based on the alignment of the target cDNAs with cDNAs from related proteins, to avoid cross-amplification

Taking into account the previous considerations the following primers (**Table 2.7**) were designed:

Table 2.7: Sequences for forward (Fw) and reverse (Rw) primers designed for qPCR using Beacon Designer (Premier, Biosoft International), complementary to the cDNA sequences of the genes encoding for the proteins Tg, CtsBa, TPO, NIS and TSH, GAPDH-2 and 18S. The amplicon (or product) length in base pairs (bp) is indicated (Primer for 18S was previously designed by a MCE group member).

Gene	cDNA Accession Number		Primer sequences (5' → 3')	Product length (bp)
Tg	DQ278875.1	Fw	TCATCAGCAGAGCCAAGAACATCAAG	177
		Rv	ATCCTGAAGGTGTGGGCGAGTG	
CtsBa	BC056688.1	Fw	CCGTCTGCTGCTTGGGATTTCTGG	104
		Rv	TGCTTCACAGGGTTCAATGGTATATGGAC	
TPO	XM_692744.3	Fw	CCAGCCAGACCTCGTTC	140
		Rv	CGGAGATGAGCGGAAGAAG	
NIS	DQ402039.1	Fw	TTTGCGATAGACCCTCAGAG	183
		Rv	GCTCACAATCAGACACAGAC	
TSH	AY135147.1	Fw	TGGTGGGTCCTCGTTTTATTGTTCAG	179
		Rv	GCGGGTTCTAAGGGCACATTCATC	
GAPDH-2	AY818346	Fw	CTGTGGGCAAAGTCATTCCTG	135
		Rv	ACTCCTTGATGTTGGCGTAGC	
18 S		Fw	CGATCAGATACCGTCGTAGTTC	
		Rv	CCCTTCCGTCAATTCCTTTA	

2.4.7.2 qPCR reactions

The qPCR reactions (20µl) were performed for each sample in duplicate reactions with 10 µl of Power SYBR® Green PCR Master Mix (2x) (Applied Biosystems, UK), 0,2 µl of each specific primer, Fw and Rv (10 µM), 2 µl of the cDNA template (diluted 1:10) and 7.6 µl of sterile milliQ water. In the case of TSH and TPO target genes, for which a lower gene expression (higher Ct) was detected, the qPCR reactions were carried out using non-diluted cDNA for each individual. The qPCR reaction in the Bio-Rad iClycler iQ5 qPCR thermocycler was initiated at 95ºC for 10 min for enzymatic activation, followed by 55 cycles of denaturation (95ºC, 30 seconds), annealing (Ta, 20 seconds) and synthesis (72ºC, 30 seconds).

After the synthesis cycles ended the melting curve was carried out by a progressive increase of the temperature in a total of 75 cycles, starting at 60 ºC for 10

min and increasing each cycle by 0.5 ºC, in order to separate PCR products by their melting curve and allow the detection of non specific products and dimers.

2.4.7.3 qPCR optimizations

For successful results using this molecular tool, a series of steps were needed before performing the final reactions as described in the previous section (2.4.7.2).

It was first necessary to optimize the Ta for each pair of primers, the sample cDNA dilutions and to prepare the miniprep dilution series used in the standard curves.

a) Ta optimization

Although in the normal PCR reactions several factors may be manipulated to achieve optimal amplification conditions, in the qPCR reactions the only parameter that was manipulated was the temperature.

The same cDNA was used to optimize all pairs of primers (a mix of cDNAs from the control group, diluted 1:10), by testing its amplification through a temperature gradient based on the Ta suggested by the software of primer design. An optimal temperature of 60ºC was chosen for all primer pairs (the temperature obtaining a lower threshold cycle and a higher fluorescence level for the same cDNA).

b) Standard preparation

The preparation of the standards consisted of **linearization of pDNA** so that its structure would be as similar as possible to the cDNA (see section 2.4.3), followed by its **purification** and **quantification**.

To linearise the minipreps, a restriction enzyme cutting at a unique site was chosen for each target by restriction analysis using the software Webcutter 2.0 (freeware [70]) genes, and following two rules: the restriction enzyme had to cut the

plasmid only once and it could not cut inside the insert sequence. The enzymes chosen and the enzyme reaction components were as presented in **table 2.8**.

Table 2.8: Enzymes and the enzyme reaction components in µl used for the linearization of the different pDNA to be used for the standard curve of each gene in qPCR. Enzymes and buffers were purchased from Promega (D, H or MC=Multi Core).

	Tg	CtsBa	TPO	NIS	TSH	GAPDH-2
pDNA (µl)	8	8	6	8	8	6
Buffer	D	MC	H	MC	MC	H
Buffer 10x volume (µl)	4	4	4	4	4	4
Enzyme	*Sal*I	*Apa*I	*Pst*I	*Apa*I	*Apa*I	*Pst*I
Enzyme volume (µl)	1.5	1.5	1.5	1.5	1.5	1.5
st water (µl)	26.5	26.5	28.5	26.5	26.5	28.5

Samples were incubated at 37 ºC for 1.5h. As the electrophoresis results confirmed the successful digestion by analysis of a small aliquot of digestion product (2 µl), the remainder of the linearised pDNA was purified using Illustra GFX PCR DNA and Gel Band Purification kit (GE Healthcare) according to manufactures instructions.

For the purification of linearised pDNA, 500 µL of Capture buffer were added to the samples. The mixture was loaded onto a purification column placed in a collection tube and centrifuged at room temperature in a microcentrifuge at maximum speed for 30 sec. The flow-through was discarded and wash buffer added to the column followed by another centrifuge step for 30 sec. The collection tube was discarded and the column transferred to a fresh DNase-free tube. 40 µl of Elution buffer were placed in the centre of the membrane, incubated at room temperature, 1 min, and centrifuged at room temperature, maximum speed for 1 min.

The linearised pDNA was quantified by preparing Quant-iT™ working solution obtained with a 1:200 diluting of Quant-iT™ dsDNA HS reagent in Quant-iT™ dsDNA HS buffer. Once the working solution was prepared, 1 µl of the samples (1:100 linearised pDNA) and 10 µl of each standard (standard 1 and 2) were loaded into assay tubes to which 199 µl and 190 µl of working solution were added, respectively. The

Qubit™ fluorometer was calibrated with the prepared standards followed by the reading of the samples concentrations.

c) Sample dilutions optimization

The standard curve was attained through the duplicate of serial dilutions, 10-fold dilutions from 0.5 ng/µl to 5.0 ag/µl, of the linearised pDNA containing the cloned target gene, and the mean used to calculate efficiency of the qPCR reaction.

The sample dilution test with cDNA dilutions also in duplicate from 1:5 to 1:3125 served to verify if the number of transcripts of each gene fit in the linear part of the standard curve and also to analyse the qPCR efficiency.

2.4.7.4 Normalization

The genes 18S and GAPDH-2 were tested for housekeeping genes using optimized primers (table 2.6) and temperature (Ta = 60.0ºC). After validation GAPDH-2 was used for samples normalization, as it presented no significant differences in expression between groups. Then, the mean amount obtained for each gene in each cDNA was divided by the mean absolute value obtained for GAPDH-2, quantified following the same procedure as the target genes.

2.5 Statistical Analysis

Statistical analyses of the results from the morphological and molecular analysis were performed using the software SigmaStat.

The results from the morphological analysis were submitted to One Way ANOVA and Tukey-test with the $\log_{10}$ of the variable (internal area, external area and thyrocyte height).

In respect to the results of the qPCR, the validation of 18S and GAPDH-2 was achieved by a One Way ANOVA and Tukey-test of the variable (Starting quantity). The

normalized results of the genes CtsBa, Tg, TPO, TSH and NIS were also submitted to One Way ANOVA and Tukey-test with the $\log_{10}$ of the variable (relative expression).

3. Results

By using a series of tools, morphological and molecular, such as PCR and qPCR, a number of results were attained during the practical work.

By the end of the 21 days of the experiment, an apparent behavioural change in the fishes treated with the bactericide TCS was detected. These animals had a different swimming pattern from the control animals and from the ones treated with PTU, but additional behaviour studies would be necessary to confirm this.

The final mean weight and length and associated standard error (S.E.M) for each group were those presented on **table 3.1**.

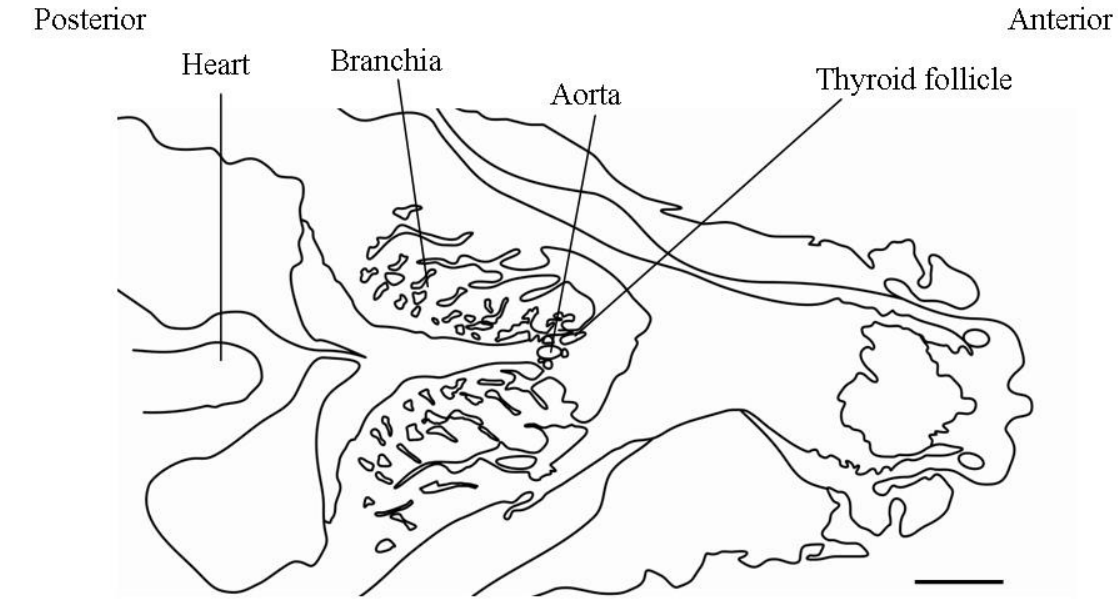
Table 3.1: Mean body weight (g) and length (cm) (**±** SEM) for the different treatment groups after the 21 days exposure to the treatments for n=8.

Tank	Mean body weight (g)	Mean length (cm)
Control 1	0.473 ± 0.04	2.938 ± 0.05
Control 2	0.532 ± 0.04	2.877 ± 0.07
PTU 1	0.424 ± 0.02	2.820 ± 0.06
PTU 2	0.460 ± 0.05	2.946 ± 0.10
TCS 1	0.484 ± 0.02	2.876 ± 0.05
TCS 2	0.566 ± 0.03	3.025 ± 0.05

3.1 Histological results

The thyroid tissue was examined in 8 individuals per experimental group ensuring that approximately the same area was surveyed in each individual. The diagram in **fig 3.1A** represents the region selected for analysis of thyroid follicles which were clustered around the aorta and the heart was evident in the section. Clear differences were observed in the appearance of the thyroid tissue stained with Cleveland-Wolfe trichrome prepared from different treatment groups (**fig 3.1B**). The staining method permitted easy identification of the thyroid follicles in histological sections and the colloid and lining thyrocytes were readily differentiated. For example, when simple stereology was applied the TCS group was found to have a higher number of follicles which were also bigger than those found in control fish. In contrast, the PTU group had a lower number of follicles than control fish. In addition, analysis at a high magnification revealed that the thyroid follicle epithelium was thinner in both TCS and PTU treated fish compared to the control fish.

A



B

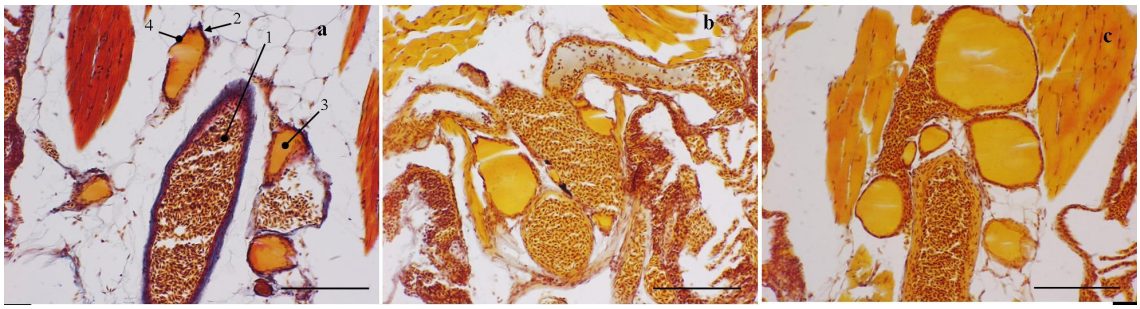


Fig 3.1: Thyroid tissue histology in treated and control fish. **A** – Scheme (photoshop rendered of a low magnification photograph) of the region in which the thyroid follicles were observed. Sections were prepared and stained with Cleveland-Wolfe trichrome and when the aorta was visible and the heart evident follicles were analysed in the relevant sections. The scale bar corresponds to 500 µm). **B** - Thyroid tissue stained with Cleveland-Wolfe trichrome from the different treatment groups, (a) - control, (b) - PTU and (c) - TCS, Note the orange staining colloid (3) in the centre of thyroid follicles (2) which are distributed in connective tissue surrounding the aorta (1). The thyrocytes (4) are evident as a layer of cells surrounding the colloid and isolate the follicle lumen from the surrounding tissue. The scale bar corresponds to 100 µm.

In order to obtain a quantitative comparison for the morphology of the thyroid tissue in the experimental groups stereology was carried out. Follicle internal and external areas as well as the thyrocyte height were measured in 4 different individuals on 6 sections and the results obtained were compared.

The mean area of the follicle lumen (internal and external) and mean thyrocyte height and S.E.M for the different treatments are presented in **fig 3.2**. Considerable variability in the area of the follicle lumen occurred in all groups and large and small follicles were evident in all of the experimental groups (**fig 3.1 B**). However, in general

the mean internal area of thyroid follicles was greater in the group of fish treated with TCS (6606±2405 μm^2) compared to the control (1913±621 μm^2) and PTU (2386±637 μm^2) treated fish. Concerning the mean external area, the TCS group stands out as the group with a higher mean value (7165±2485 μm^2) when compared to the control (2417±730 μm^2) and PTU (2791±674 μm^2) groups.

To evaluate if the differences observed between the groups were significant, data were then subjected to statistical analysis (One Way ANOVA) after $\log_{10}$ transformation of each variable. A significant statistical difference among groups ($P = 0.035$) was detected for the internal area measurements, and using Tukey test for pairwise comparison between groups, a statistical significant difference ($P = 0.05$) was detected between the TCS and the control groups. The same type of statistical analysis detected statistically significant differences ($P = 0.05$) in the follicle external areas between groups (**fig 3.2**), with the TCS group being significantly greater than the control and PTU groups which did not differ significantly.

A difference in the mean thyrocyte height was also observed between groups, with highest values found for the control group (2.77±0.198 µm) followed by the PTU (1.708±0.0728 µm) and TCS (1.618±0.0733 µm) groups (**fig 3.2**). Statistically significant differences between the height of thyrocytes between groups ($P < 0.001$) were confirmed by One-way ANOVA. This test was followed by a Tukey test, indicating statistically significant differences existed between TCS *vs* Control and PTU *vs* Control (both with $P < 0.001$).

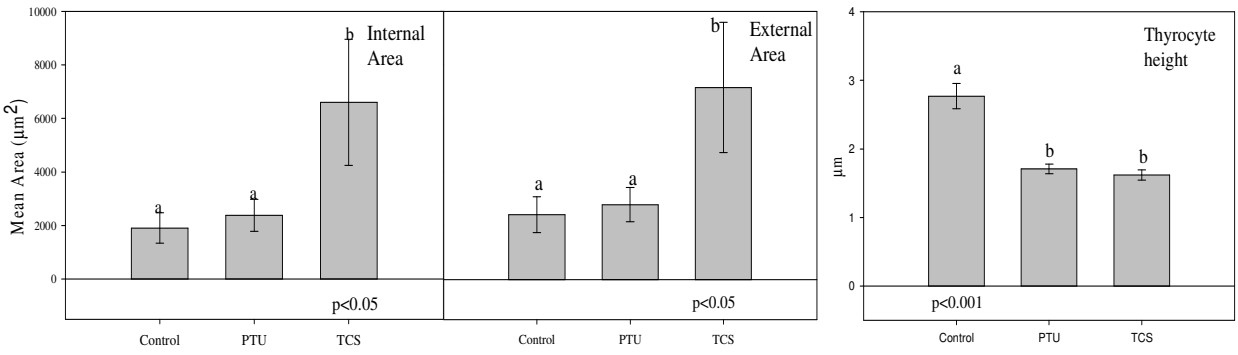


Fig 3.2: Graphical representation of the mean ± SEM of thyroid follicle internal/ external areas (μm^2) and mean thyrocyte heights (µm) determined for fish treated for 21 days with the goitrogen PTU (n = 4), TCS (n = 6) and control (n = 4). The different letters (a and b) indicate statistically significant differences exist between groups in each panel.

3.2 Molecular Results

As a complement to the histological and stereological analysis molecular analysis of specific gene expression was assessed by qRT-PCR. The genes selected for analysis were those encoding proteins known to be of key importance in THs biosynthesis in the thyroid follicle. The analysis was conducted with whole-heads of zebrafish, ensuring that the thyroid tissue was present and the effect of exposure to test chemicals was evaluated by measuring their mRNA expression.

3.2.1 Tissue gene expression

In order to confirm the gene expression and relative expression levels of the genes in study in the head of zebra fish, RT-PCR reactions were performed with specific primers (cloning, see table 2.1, methods) for each gene in optimized conditions (see table 2.3, methods,) . Bands of the expected size were obtained by RT-PCR for all the genes of interest (**figure 3.4**), as expected, confirming their expression in the tissues from which the RNA was extracted. These cDNA fragments were then cloned and sequence analysis used to confirm their identity.

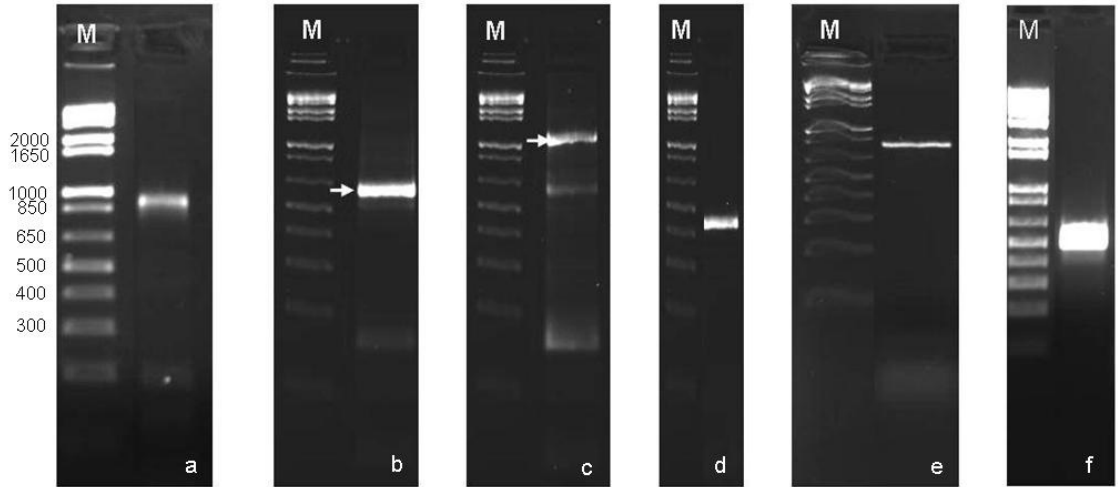


Fig 3.2: RT-PCR products with gene specific primers from zebrafish cDNA (head) in 1.5% agarose gel stained with ethidium bromide, using optimized conditions. The amplified genes were (a) **TPO** - Ta = 60ºC, [$MgCl_2$] =1mM and 40 cycles; (b) **CtsBa** - Ta = 59,3ºC, [$MgCl_2$] =2mM and 30 cycles; (c) **NIS** - Ta = 52ºC, [$MgCl_2$] =2,5mM and 30 cycles, (d) **TSH** - Ta = 63,4ºC, [$MgCl_2$] =2mM and 30 cycles and (e) **Tg** - Ta = 60,4ºC, [$MgCl_2$] =2mM and 30 cycles and (f) **GAPDH-2** - Ta = 60ºC, [$MgCl_2$] =1mM and 40 cycles. Sizes of the bands were compared with the sizes of the 1 Kb Plus DNA Ladder (Invitrogen) (M), which are indicated in base pairs (bp) in the left panel. For the genes CtsBa and NIS, (b and c) the PCR reactions resulted on several fragments; the fragments with the expected size are the most intense and are signalled with the arrow head.

3.2.2 Validation of internal reference genes for qRT-PCR and assay characterization

qRT-PCR efficiency was determined using the relative standard curve method with serial dilutions of the cloned and sequenced cDNAs for the desired gene and with a cDNA dilution series (from 1:5 to 1:3125) of control samples for the target genes (**table 3.1**). cDNA dilutions 1:10 were also used for the specific real-time primers and provided a first approach to establish the Ct values of each gene (**table 3.1**).

Table 3.2: qRT-PCR efficiencies for the different genes determined for the standard curve (with dilution series from 0.5 ng/µl to 0.5 fg/µl of cloned cDNA) and for the cDNA dilution series (1:5 to 1:3125). Ct values for the control cDNA for an automatically defined threshold.

		qRT-PCR efficiency (%)			
Gene	**C_T** (cDNA dilution 1:10)	**Standard curve**	**r^2**	**cDNA**	**r^2**
Tg	25	88,7	0,998	81,4	0,998
CtsBa	26	104,1	0,999	112,3	0,987
TPO	31	91,8	0,999	94,4	0,999
TSH	31	96,2	0,999	72,4	0,998
NIS	32	94,2	0,979	101,4	0,965
18S	13	85,6	0,995	79,9	0,977
GAPDH-2	25	83,4	0,998	104,9	0,999

The amplification efficiencies for qRT-PCR were, in general, within the recommended values for a good and sensitive quantification (efficiency higher than 80% and R^2>0,985 [71]). Most of the target genes gave a similar efficiency for amplification with cDNA as the template and in standard dilutions of linearised cloned cDNAs. However, in some cases, such as TSH, the amplification efficiency was lower when cDNAs generated from tissue were used. This may have been caused by the very low amount of material in the samples applied in the reactions (as is the case of TSH) or by the sample with the higher dilution factor from the cDNA dilution series that with a very weak signal (in 18S), affected the overall efficiency. The Ct values obtained for a 1:10 dilution of control cDNA was used to give a first indication of the level of expression of each gene and formed the basis of the selection of the dilutions for use in quantification. It was decided to use for sample quantification a 10 fold dilution for

most of the genes, with moderate to high genes expression (eg. Tg, CtsBa, TPO and GAPDH-2), and to use non-diluted cDNAs in the quantification of NIS and TSH, because of their lower gene expression (reflected by their higher Ct values).

3.2.2.1 Reference gene validation

In this work the genes 18S and GADPH-2 were assessed as possible reference genes prior to their application. The level of expression of each gene was evaluated between the treatment groups, using cDNA synthesised from equal amounts of RNA extracted from experimental animals to be used for qRT-PCR. For validation, the starting quantity (SQ) for each gene was submitted to a One Way ANOVA test followed by a Tukey test if possible (**fig 3.5**). Analysis of the results revealed that the gene 18S was unsuitable for use as a reference gene as it presented statistical significant differences in the mean SQ between the control (0.153 ± 0.026 ng) and TCS (0.034 ± 0.0073 ng) groups (**fig 3.5**). The mean SQ of the PTU group (0.095 ± 0.018 ng) presented no statistical significant differences from the remaining groups.

For GAPDH-2, **fig 3.5**, no statistical significant differences between the groups control ($2.34x10^{-5} \pm 6.25x10^{-6}$ ng), PTU ($2.48x10^{-5} \pm 6.98x10^{-6}$ ng) and TCS ($1.54x10^{-5} \pm 1.20x10^{-6}$ ng) were found, and although a small apparent decrease is observed in the TCS group. Thus, the gene GAPDH-2 was validated as a reference gene for the qPCR results normalization.

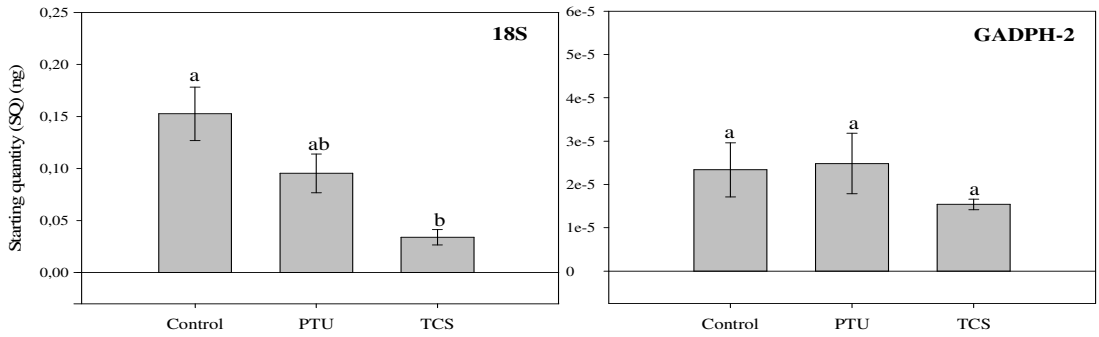


Fig 3.3: Mean starting quantities (ng) for 18S and GAPDH-2 genes for the different treatments (Control n =5, PTU n = 6 and TCS n = 5). The different letters (a and b) are indicative of significant statistical differences (P <0.05) between the treatment groups, evaluated by a One Way ANOVA of the variable, followed (for 18S) by a Tukey test.

3.2.2.2 Changes in Gene Expression

The expression of each of the genes was then quantified in the individual samples of zebrafish heads under the different treatments using optimized qRT-PCR conditions (see section 2.4.7.2). The relative expression for the different samples and different genes was calculated by the ratio between the starting quantities obtained for the gene of interest and for the validated reference gene, GAPDH-2. Group means for the relative expression of all genes analysed are presented in **fig. 3.6**. A statistically significant difference ($P < 0.05$) was found in the expression of TSH in the control ($5.16x10^{-4}\pm1.74x10^{-4}$) versus the TCS ($2.29x10^{-3}\pm8.86x10^{-4}$) and PTU ($2.00x10^{-3}\pm5.43x10^{-4}$) treated fish, and in the latter groups it was significantly increased ($p<0.05$). The high levels of TSH observed in the PTU group are in agreement with induced hypothyroidism, where lower THs in circulation increases the level of expression of TSH. These results are as expected for the PTU group as this drug is known to inhibit intrathyroidal synthesis of thyroid hormones (by influencing TPO activity) and blocks the peripheral conversion of T_4 to T_3, thus lowering THs levels.

The level of expression of the gene NIS in the TCS group was also significantly different ($P < 0.05$) from the control fish or those treated with PTU. For this gene, the TCS exposed fish had a higher level of relative expression ($3.52x10^{-3}\pm6.57x10^{-4}$) than the control ($1.61x10^{-3}\pm4.42x10^{-4}$) and PTU ($1.98x10^{-3}\pm2.10x10^{-4}$) groups.

Analysing the relative expression dispersion in **fig. 3.6** (dots), it can be noticed, an internal variability in gene expression in some of the treatment groups. In the results for TPO relative expression, the control group revealed high data dispersion indicating variable expression relative to the treated fish. Of all the remaining genes analysed, the highest variability of expression was found in the TCS exposed fish.

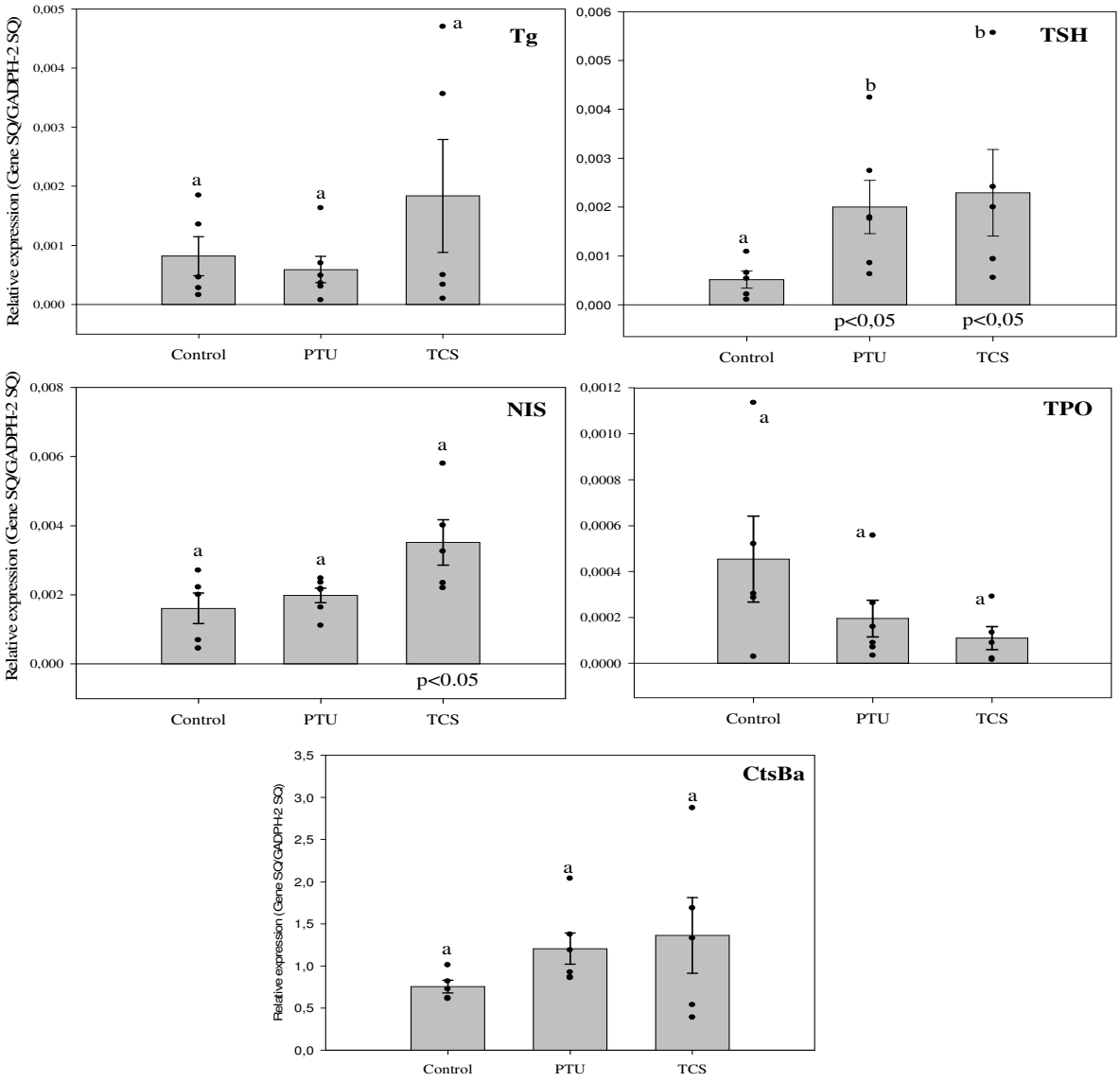


Fig 3.4: Dispersion (dots) and mean relative expression (gray bars) of the several genes analysed (Tg, TSH, TPO, NIS and CtsBa) normalized with GAPDH-2 (SQ gene/SQ GADPH-2) for the treatments control (n = 5), PTU (n = 6) and TCS (n =5) and respective SEM (bars). The different letters (a and b) are indicative of significant statistical differences (P <0.05) between the treatment groups, evaluated by a One Way ANOVA of the $\log_{10}$ variable, followed by a Tukey test for TSH and NIS.

4. Discussion

The antimicrobial agent TCS, widely used in consumer products, is now an established environmental contaminant [72]. Although it is known from the work by Crofton *et al* that TCS decreases the level of THs in circulation in mice [47], its effects at the level of thyroid hormone synthesis are not known. The results in the present thesis indicate that TCS leads to morphological changes in the thyroid tissue, such as follicle size increase and decreased thyrocyte height in a fish model, the zebrafish. The effects of TCS on the thyroid tissue were also found at the gene expression level. The TSH and NIS genes presented expression levels significantly higher for the TCS group than those observed for the control group, in zebra fish head. For the remaining genes analysed (Tg, TPO, and CtsBa), apparent differences were observed compared to the control group, but did not reach statistical significance because of the spread of the data in the treatment group. The final results for the group treated with PTU, here used as a positive control since it is a goitrogens whose effects are most studied; indicate morphological alterations, with the reduction of thyrocyte height, as well as changes in the levels of gene expression. In PTU treated fish the TSH gene presented significant increase in expression while the remaining genes were not changed.

Since the exposure to some drugs (therapeutic or industrial pollutants) in high quantities may cause goitrous hypothyroidism [8] and has been described mainly in mammals and is diagnosed by low circulating TH concentrations with enlargement of the thyroid tissue. In the present study the thyroid activity of zebrafish exposed to TCS and PTU was evaluated by histological and stereological analysis, in order to assess the effects of these drugs.

It has been described that inactive thyroid tissue possess flat cells (thyrocytes) lining the follicles and, in contrast, active follicles present cuboidal thyrocytes [73]. The observations of zebrafish thyroid tissue (fig 3.1 B) in the present work suggest that the individuals exposed to TCS and PTU presented inactive thyroid follicles. Our stereological analysis confirmed that a significant reduction in the follicle thyrocyte height in the PTU and TCS groups occurred (fig 3.2), indicating the inactive state of the follicles.

The histological and stereological results of the tissues from individuals treated with TCS presented the most significant changes. Alterations in thyrocyte height and

mean follicle areas (internal and external) were found to be lower and higher, respectively, when compared with the control group (fig 3.2). The increase in follicle areas is due to colloid accumulation and is also described as a characteristic of hypothyroidism [73]. Results in the thyroid tissue from the zebrafish treated with PTU, indicative of thyroid inactivation, are also in accordance with a situation of hypothyroidism, as described by Villar *et al* for PTU-treated goats [52]. van der Ven *et al* also described the reduction of THs in the circulation in adult zebrafish exposed to PTU [23] indicating hypothyroidism was induced. In our PTU treatment group no significant increase in follicle area was observed. Results were different from the observations of Yi *et al*, in rat, where the follicular colloid increased with the administration of PTU [51] and from van der Ven *et al* who observed an increase in follicle activity in juvenile zebrafish [23].

A reduction of THs in the circulation in response to TCS treatment has been previously reported in rats [47]. Although it was not possible to measure the TH levels in this study, due to the small size of fish, the data acquired strongly suggest that TCS affects TH synthesis, causing hypothyroidism, as evidenced by histological and gene expression changes characteristic of this status.

The expression analysis of the mRNA levels of key genes related to TH synthesis was performed using quantitative RT-PCR technique, using the relative standard curve method with normalization to a reference gene. With this approach the errors (differences in mRNA input or RT-efficiencies between samples) possibly introduced in the steps performed prior to qRT-PCR are controlled [62].

However, the choice of reference or internal control genes is not consensual. While many studies use only one reference gene, it has been suggested (Vandesompele *et al*) that this approach leads to relatively large errors and that accurate normalization can only be attained by carefully selecting multiple reference genes for each particular experiment or system [69]. In this study, the normalization of the expression of the genes of interest was performed using GAPDH-2, which in our validation assay (fig 3.3) presented no statistically significant differences between the treatment and control fish, using samples synthesised from the same amount of RNA. Our results in GADPH-2 expression were found to be in accordance to the work from Manchado *et al*, where expression levels in Senegalese sole larvae remained unaltered even when animals are exposed to a blocking agent of THs synthesis (thiourea) [74].

The gene 18S rRNA was also evaluated as reference gene, since it is one of the most frequently used reference gene which is found to be invariant in many biological situations [62]. However, after validation it was detected that its gene expression had a significant variation between the groups (fig 3.3), thus making the gene inadequate for this study.

This validation results suggest that the goitrogen treatment is somehow affecting protein synthesis, as the expression of the 18S rRNA (a ribosome unit) is significantly reduced in the TCS-treated group, while the glycolitic processes are probably not significantly affected by the treatment (GAPDH-2 levels are not significantly affected).

For each gene analysed, some variability of response between the animals of the same group can be observed (fig 3.4, dispersion graphs) making possible the detection of some extreme responses in some of the animals, while in others the expression levels are unaltered. The observed variability may be due to variability between the responsiveness of individual fish, which could be reduced by increasing the number of fish tested, or to differences in the dose consumed (orally) by the different fish.

The expression of the key genes involved in TH synthesis (Tg, TSH, TPO, NIS and CtsBa) was analysed in whole zebrafish heads, which was considered to be a good approximation to their expression in the thyroid tissue (difficult to isolate due to small fish size and dispersed characteristic of thyroid tissue in fish) since the expression of most of these genes are thyroid (Tg, NIS, TPO) and pituitary-specific (TSH) [10]. Only CtsB is not thyroid specific but possesses an important role in colloid solubilisation. In the absence of CtsB there was a reduction in T4 circulating levels in mice [22].

When analysing the mRNA relative expression results (fig 3.4), significant increases in the TSH (in both treatments) and NIS (only in the TCS treatment) levels can be observed. For the genes Tg, TPO and CtsBa, slight variations were detected which were not statistically significance between treatment and control fish.

Pituitary TSH synthesis is known to be regulated by the TH levels, and their circulating levels in turn regulate THs synthesis and liberation (HPT axis). Pradet-Balade *et al* have studied the relation between THs and TSH mRNA levels *in vivo* in the turbot. For diminished levels of T_4, TSH levels were increased [75]. These conditions were representative of hypothyroidism and demonstrated that THs regulate TSH by negative feedback in order to maintain THs levels constant.

In this study, the TSH gene relative expression in zebrafish heads increased in the treatment with PTU. This drug is known to interfere with the normal functioning of

the enzyme TPO and to inhibit peripheral conversion of T_4 into T_3 [8]. In previous studies in goat and rats, PTU was shown to induce hypothyroidism, by the observed reduction of circulating T_4 levels. In the study by Hood *et al*, T_4 reduction was accompanied by an increase in TSH levels [47, 76].

In our experiment, the TSH relative expression was also significantly increased in the TCS group when compared to the control. It is known from the work by Crofton *et al*, in mice, that T_4 levels were reduced for animals treated with TCS [47], at the same TCS dosage used in this thesis. Considering these results and previous work on the effects of PTU, the fact that TCS was able to induce an increase in TSH gene expression suggest that this compound may also have caused a reduction in TH circulating levels and a consequent increase in TSH mRNA and protein production and release, in order to restore TH levels. However, further work is needed to confirm this observation.

TSH levels are known to regulate the expression of several genes involved in TH synthesis, such as Tg, TPO, NIS and CtsB, in order to maintain homeostasis of the HPT axis. Caraccio *et al* showed that higher concentrations of TSH lead to higher mRNA expression levels of NIS, TPO and Tg, in human thyrocyte cultures [77]. TSH positive regulation of NIS expression (the increase of TSH induces the increase of NIS) was also confirmed *in vivo* in rats and humans [78, 79].

Based on previous reports and on the observed increase in TSH mRNA expression levels, it should be expected that an up-regulation would be found in the other genes analysed in response to PTU and TCS treatment. A significant increase in NIS expression was indeed found for the TCS group, but not for the PTU group (fig 3.4). This suggests that TCS also influenced the uptake of iodide into thyrocytes, by increasing the expression of this transporter, possibly to overcome the reduction of TH levels suggested by increased TSH expression and histological analysis. Whether this effect of TCS on NIS expression levels is direct or mediated by the change in TSH levels is not known, but the fact that no increase in NIS expression was observed for the PTU group despite the increase observed in TSH expression levels suggest that the effect may be direct. It is also possible that an increase in NIS expression levels occurred in the PTU group in an earlier time-frame, and then stabilized to the levels measured after 21 days of treatment.

In respect to the relative expression levels for the enzyme TPO, responsible for iodide oxidation and iodination of the TH precursor Tg, high variability was found

between individuals (especially in the control group) and no significant differences were detected between treatment groups (fig 3.4). However, an apparent decrease in both the variability and the level of expression was observed in the PTU group and, more pronounced, in the TCS group. Although not significant, the suggested decrease in TPO expression contrasts with previous studies associating low TPO expression levels with the reduction in TSH levels, in human thyrocyte cultures and, in vivo, in normal and tumoral human thyroid tissues [77, 80, 81]. Whether the observed differences are due to species- or time-course differences is not known and needs to be further explored.

In addition, the goitrogen PTU is known to produce its anti-thyroidal effects by interfering with TPO-mediated iodine utilization in thyroid follicles in mammals. Although PTU is used to induce hypothyroidism also in fish [23, 51] no information was found about the effects of this goitrogen on the expression of TPO in fish.

The results obtained in this thesis for the mRNA expression levels of Tg did not present significant differences on the gene expression between treatment groups. This contrasts with previous studies showing the increase in Tg mRNA expression in response to increased TSH concentrations in human thyrocyte cultures, and in rat thyroid gland in response to PTU treatment [51, 77]. Nevertheless, an apparent (but not statistically significant) increase in Tg mRNA expression was observed in the TCS group , which is caused by two individuals that present an extreme response with a great increase in Tg expression (fig 3.4). Clearly it will be important in the future to repeat the analysis with samples taken at different time points and also with a greater number of samples to conduct a more robust analysis of the effect of TCS on Tg expression.

CtsBa is an isoform of a proteolytic enzyme (CtsB) that, although involved in the liberation of THs from Tg, and is not restricted in expression to thyroid tissue [10], which could explain its high expression levels obtained for all the groups when compared to the other genes analysed. A slight increase in CtsBa mRNA expression was observed for the same groups where TSH levels were increased (the PTU and TCS groups), but the increase was not statistically significant. These observations are in accordance with the detection of increased CtsB mRNA expression in rat thyroid cells, *in vitro*, in response to increased of TSH [82]. However, the relative expression of CtsB observed in fig 3.4 reflects the global response of all tissues in the head section, and not only from the thyroid tissue. To assess the expression of mRNA encoding for this gene

only in the thyroid, further studies are required, by qRT-PCR on smaller sections or by localization techniques such as *in situ* hybridization.

In the present work, different responses were observed for the two compounds tested. The response to the (predicted) hypothyroidism induced by PTU did not present the characteristic enlargement of the thyroid follicles [73], but caused a decrease in thyrocyte height indicative of low thyroid activity. Although the expected increase in TSH levels occurred, these were not accompanied by the increase in expression of other genes involved in TH synthesis. This situation could be representative of thyroid tissue inactivation by PTU in such a way that the high levels of TSH expression could not surpass PTU concentration in circulation and maintain hormonal homeostasis. Another explanation for the difference between the observations and previously described results is that PTU in widely used and studied, in mammals, but almost no information for its action is found in fish.

In the TCS treatment, evidence for hypothyroidism were observed as indicated by the characteristic follicle enlargement, reduced thyrocyte height, as well as the increase of TSH expression levels. The expression levels of the other analysed genes did not present a significant increase with exception to NIS, the iodide pump. The increase in NIS expression may be a compensatory mechanism to overcome the reduction in circulating THs, regulated by TSH increase. This increases the iodide availability for TH synthesis. A similar increase in NIS expression is also observed in response to low iodine levels, to increase iodide uptake and compensate for the lower serum concentration [10].

It has to be taken into to consideration, when analysing the results presented in this thesis, that the expression levels determined correspond to only one point in time (after 21 days treatment) and that in this point the thyroid system could have reached a new equilibrium; also the relative expression levels studied correspond to the transcriptome and not to the proteome (protein levels in the tissue), that could show different tendencies as proteins are more stable then mRNA and are present for a long period of time. In order to further detail the responses involved in the exposure of adult zebrafish thyroid tissue, additional experiments using a higher number of individuals and studying earlier time points should be performed in the future.

Endocrine disruption is caused by compounds that alter normal hormone regulation (endocrine disruptors). It has become a major environmental issue as strong evidence for the effects in wildlife has become more noticeable. The effects observed include development disruption, altered reproductive capacity and abnormal behaviours [83]. One of the environments most affected and studied for the actions of endocrine disruptors is the aquatic environment, into which more than 100000 different chemicals are discharged directly [35]. The effects of the EDs in this environment are more pronounced as any lipophilic contaminants present in the water surrounding fish or amphibians are readily absorbed, making these animals very susceptible to these compounds action. Bioaccumulation effects also need to be taken into consideration as most EDs are lipid-soluble and so are concentrated into the fat of the animal [83, 84].

Chemicals detected as potential EDs include polychlorinated biphenyls, steroidal oestrogens (both natural and synthetic), alkylphenol polyethoxylates (used as surfactants), various plasticizers, and a range of herbicides and pesticides. Pharmaceuticals discharged in the aquatic environment might also contribute to endocrine alterations in the wildlife [83]. Among the pharmaceuticals released into the environment, TCS can be found. The widespread use of TCS in PCPs leads to a significant presence of this compound in sewage systems in the influent, effluent as well as in the sludge produced. Concentrations in the range of 0.01-0.65 μg/l in sewage effluent and 0.4-12 μg/g in sludge has been recorded [38-40, 43]. The main source of the TCS found in the sewage come from the household products, like cutting boards, and PCPs-like toothpaste, mouthwashes, soaps and deodorants [48]. The amount of TCS used as a preservative in PCPs is permitted up to concentrations of 0.3% of the product. TCS is an antibacterial agent that works by blocking the active site of the bacterial enoyl-acyl carrier protein reductase enzyme, essential to fatty acid synthesis [85]. Due to its specific bacterial target TCS was though not to have any specific effects on higher species. To study the effects of TCS as an endocrine disruptor some studies have been performed. Results from the work by Crofton *et al* in mice demonstrated that TCS decreases the levels of T_4 in circulation, while Veldhoen *et al* analysed the effects of TCS on tadpoles, indicating the interference of this compound with TH-mediated gene expression (thyroid hormone receptors) during metamorphosis [47, 86]. Others (Adolfsson-Erici *et* al) have detected TCS in human breast milk and also in fish [72], suggesting the high tendency of this compound to bioaccumulate. On the other hand, the work from Canosa *et al* suggested that food samples might be contaminated by direct

contact with TCS-containing kitchenware [87], indicating one other possible route of direct exposure in man.

The results of the present study has shown that this compound does in fact induce changes on the thyroid tissue physiology of adult zebrafish, evidenced by an increased follicle area, decreased thyrocyte height and increased expression of TSH and NIS, which suggest that TCS is able to induce hypothyroidism in adult zebrafish. Taking into consideration that TCS is released into the environment by sewage effluents, it might be expected that wildlife could be strongly affected, due to direct exposure, especially affecting fish and amphibian, or indirectly, as TCS bioaccumulates in the organism. Exposure of humans due to the direct (cumulative) exposure to very low amounts of this compound and the ingestion of animals in which the compound has bioaccumulated must also be taking into consideration, and further studies should be performed to evaluate its actions in the human thyroid physiology.

5. Conclusion

This study investigated the effects of adult zebra fish exposure to the compound TCS. Histological observations allowed the direct visualisation of some of the effects: evidence of thyroid follicle proliferation and inactive follicles, which appeared as large follicles completely filled with colloid and limited by a thin epithelial layer.

Also by histological analysis, it was possible to verify that the PTU group which served as a treatment control as it reduces thyroid tissue activity, also presented evidence of inactive follicles (verified by the thyrocyte height). This indicates that the concentration of PTU applied is appropriate to induce hypothyroidism in adult zebra fish.

Molecular techniques were used as an approach to understand how the drugs acted on the gene expression of key proteins involved in TH synthesis, in zebra fish heads. Using qRT-PCR, it was observed that 18S rRNA could not be used as a reference gene and instead GADPH-2 was validated as reference gene for the zebra fish collected from the experiments, as this gene presented no variation between the PTU, TCS or control groups. Following normalization of qRT-PCR results, the relative expression of the target genes analysed indicated that only TSH was significantly affected by the PTU treatment, showing an increase in expression, while relative expression of TSH and NIS were significant increased in zebra fish treated with TCS.

In face of these results it can be concluded that the antimicrobial agent TCS at the dose used in the present study 100 mg/Kg/day appears to have a direct effect on diverse genes the protein products of which influence THs synthesis in adult zebra fish.

Although these results are highly indicative of the effects of exposure to TCS, further work could be performed as complement. Among the possibilities of analysis which would enrich the current analysis are:

- imunohistochemistry of some of the studied proteins to localize the expression and to confirm the effects at the protein level;

- analysis of gene expression at several points in time, during the 21 days, to observe the change in effect of treatment with time;

- analysis of the response of exposure to lower concentrations of TCS and also the response to the removal of the goitrogen from the environment after a previous exposure;

- measuring THs levels by the end of the treatment.

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Annex 1: Solutions

Loading Buffer

Prepare a solution of 40% glucose in sterile water (40 g glucose in 100 mL). When the glucose is dissolved, just a sprinkle of Bromophenol blue was added to give the colour to the sample. This was made tanking into consideration that only a small amount of bromophenol blue is needed, otherwise the solution would turn very blue.

Once all the components were dissolved, the solution was filter sterilise with a syringe coupled to an adapter filter of 0.2 µm. The solution was stored in a new falcon tube.

DEPC water

For 2 litters:

A dark glass bottle was filled with 2L of distilled water, to which were added 200 µL of Diethyl Pyrocarbonate – DEPC (100 µL of DEPC per litter of water)

The mixture was agitated and left to rest for 24 hours. The solution was autoclaved.

10X OPA buffer

(100 mM Tris-acetate pH 7.5 / 100 mM Magnesium-acetate / 500 mM potassium acetate)

To prepare 10 mL

Add 500 µL of TAE 50x

Weigh 0.2 g MgAc

Weigh 0.5 g KAc

Complete the volume to 10 mL with sterile distilled water. Agitate and dissolve the solids.

Make aliquots of 100 µL and store at -20 ºC.

LB (Luria-Bertani) Broth

Dissolve 4 LB broth tablets (Sigma) in 200 mL of distilled water, autoclave and store at room temperature.

- LB agar

Dissolve 8 LB agar tablets (Sigma) in 400 mL of distilled water, autoclave and allow to cool to 55 ºC in a water bath and pour into Petri dishes. This step should be performed in the laminar flow cabinet. In alternative store the bottle after autoclaving and melt in the microwave when needed, allow to cool to 55 ºC and pour into Petri dishes.

- LB selective plates

After melting the LB agar and allowing to cool to 55 ºC, add antibiotic or X-Gal to the desired concentration as presented on **table A1** before pouring into Petri dishes. This step should be performed in the laminar flow cabinet. Store the plates in the fridge for at least 2-3 h before use.

Table A 1: Antibiotic and solutions concentrations for the LB selective plates

Antibiotic/solution	**Used at**
Ampicilin	75 µg/mL
IPTG	0.5 mM
X-GAL	80 µg/mL

Stock IPTG (0,5M)

Dissolve the IPTG in water until achieve the desired concentration (0,5M)
Aliquot and store at -20ºC

Solutions for the alkaline lysis

- GTE buffer

For 500mL:

Add 4.505 g glucose, 12.5 mL TRIS-HCl 1 M (pH = 7.5) and 10 mL EDTA 0.5 M. Complete the volume with distilled water. Mix well and autoclave.

- NaOH 0,2 M/SDS 1%

For a volume of 14 mL:
Add 560µL NaOH 5M, 700 µL SDS 20% and 12.74 mL sterile water to complete the volume.

- Potassium Acetate (KAc)

- KAc 5M stock (for 200 mL):
  Dissolve 98.14 g of potassium acetate in 200 mL of distilled water, autoclave and store at room temperature.

- KAc for alkaline lysis (3M in potassium, 5M in acetate) for 100 mL:
  Mix 60 mL of KAc 5M stock with 11.5 mL glacial acetic acid. The pH was checked, to verify if it fits between 4.8 and 5.2. If not, pH was adjusted by adding more glacial acetic acid. The final volume was adjusted with distilled water and the solution was stored at room temperature.

Annex 2: APES treatment of slides

APES coating of slides is an alternative method that makes the slides more adhesive for fixed tissues and living cells.

The slides were loaded onto big slide racks, and each rack was immersed for 30 min in 1% acid/alcohol (1% v/v concentrated HCl, 70% ethanol, 29% water) thus allowing to clean the slides. After the immersion in 1% acid/alcohol the slides were rinsed in running tap water, followed by immersion in distilled water. The slides were allowed to dry ON at room temperature.

The racks with the slides were immersed for 10 min in acetone, followed by a 5 min immersion in 2% (v/v) APES in acetone. The slides were immersed briefly in 2 sequential rinses of distilled water and allowed to dry. Once dried, the slides were removed from the racks and stored in a dust-free container.

Annex 3: Competent bacteria

To obtain competent bacteria cells, one colony of the strain *Escherichia coli* XL-1 Blue, was isolated from a plate of LB medium (30 mg/ml tetracycline) and used to inoculate 5 ml of LB medium. Cells were incubated for 12-16 h at 30ºC and 250 rpm.

1 ml of the culture was inoculated in 250 ml of **SOB** medium and incubated with agitation at 18ºC until a DO of 0.6 nm was reached. The bacterial suspension was incubated on ice to stop growth for 10 min and transferred to 50 ml Falcon tubes and centrifuged 1200 g, 10 min, 4ºC. The supernatant was discarded and the pellet ressuspended in 16 ml of cold transformation buffer (TB), a rich solution of $CaCl_2$, incubated on ice for additional 10 min and centrifuged as described above. DMSO (cryo-preserving agent) was added until a final concentration of 7% was reached and bacteria were incubated for 10 min and aliquoted in 100 µl samples and immediately frozen in liquid nitrogen and stored at -80ºC until further use.

SOB médium (250 mL):
2% Bacto tryptone (5g);
1.5% yeast extract (1.25g);
10 mM NaCl (500 µL stock 5M);
2,5 mM KCl (625 µL stock 1M);
10 mM $MgCl_2$ (2.5 mL stock 1M);
10 mM $MgSO_4$ (2.5 mL stock 1M);
Autoclave after prepare.

TB buffer (100 mL):
10 mM Pipes (0.30 g);
15 mM CaCl2 (1.5 mL stock 1M);
250 mM KCl (25 mL stock 1M);
Adjust pH to 6.7 with KOH 1M, other wise the pipes will not dissolve (take in consideration that the pH changes very fast when near the optimum). After is fully dissolved and the pH is at 6.7 add 55mM $MnCl_2$ (0.69 g). Filter with 0.22 µm filter to sterilize. Do not autoclave the solution. Store it at 4 ºC until use.

Annex 4: pGEM-Teasy restriction map

pGEM-T Easy vector

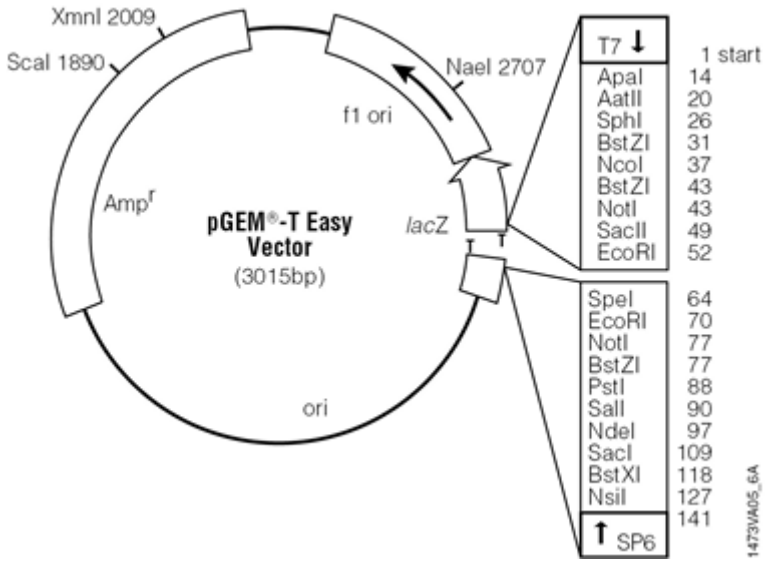


Figure A 1: pGEM -T Easy vector map and reference points.

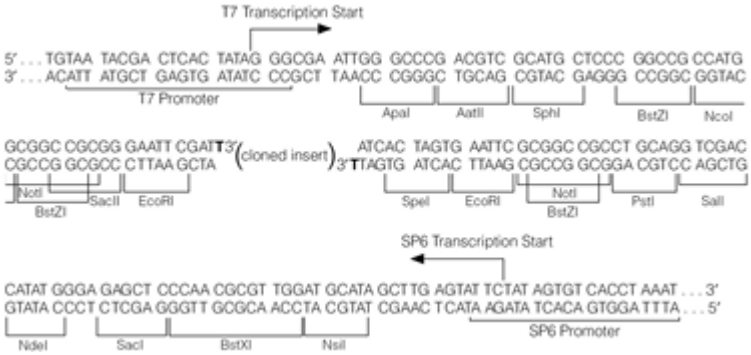


Figure A 2: The promoter and multiple cloning sequence of the pGEM®-T Easy Vector. The top strand of the sequence shown corresponds to the RNA synthesized by T7 RNA polymerase. The bottom strand corresponds to the RNA synthesized by SP6 RNA polymerase.

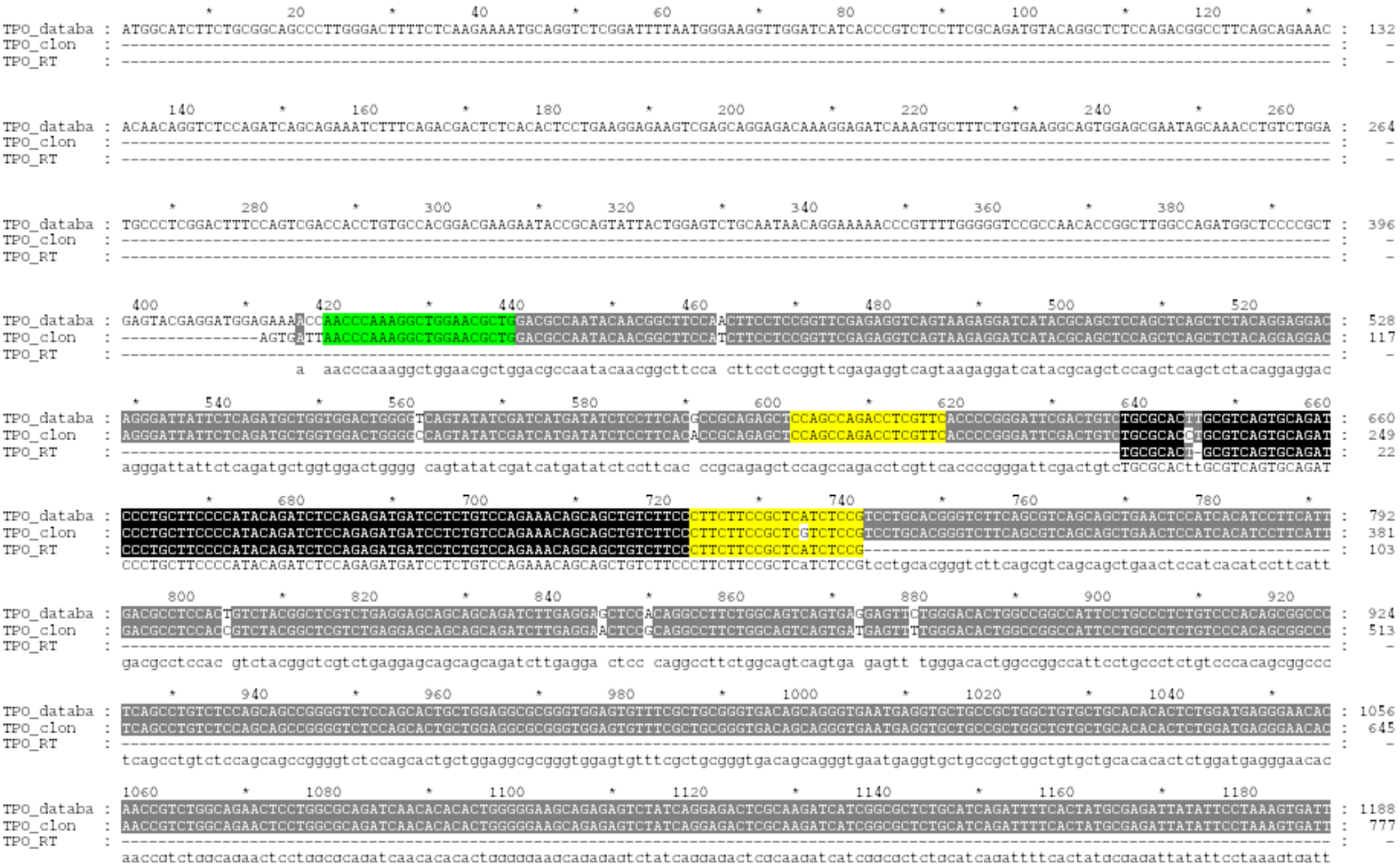
Annex 5: NIS alignment

Figure A 3: NIS sequence alignments of the sequences from the database (NIS__datab), the sequence from the cloned fragment (NIS__seque) with the cloning primer sequence (green) and the qRT-PCR amplicon sequence (NIS_RT) with the primer sequence (yellow).

Annex 6: TSH alignment

Figure A 4: TSH sequence alignments of the sequences from the database (TSH__datab), the sequence from the cloned fragment (TSH__seque) with the cloning primer sequence (green) and the qRT-PCR amplicon sequence (TSH_RT) with the primer sequence (yellow).

Annex 7: TPO alignment



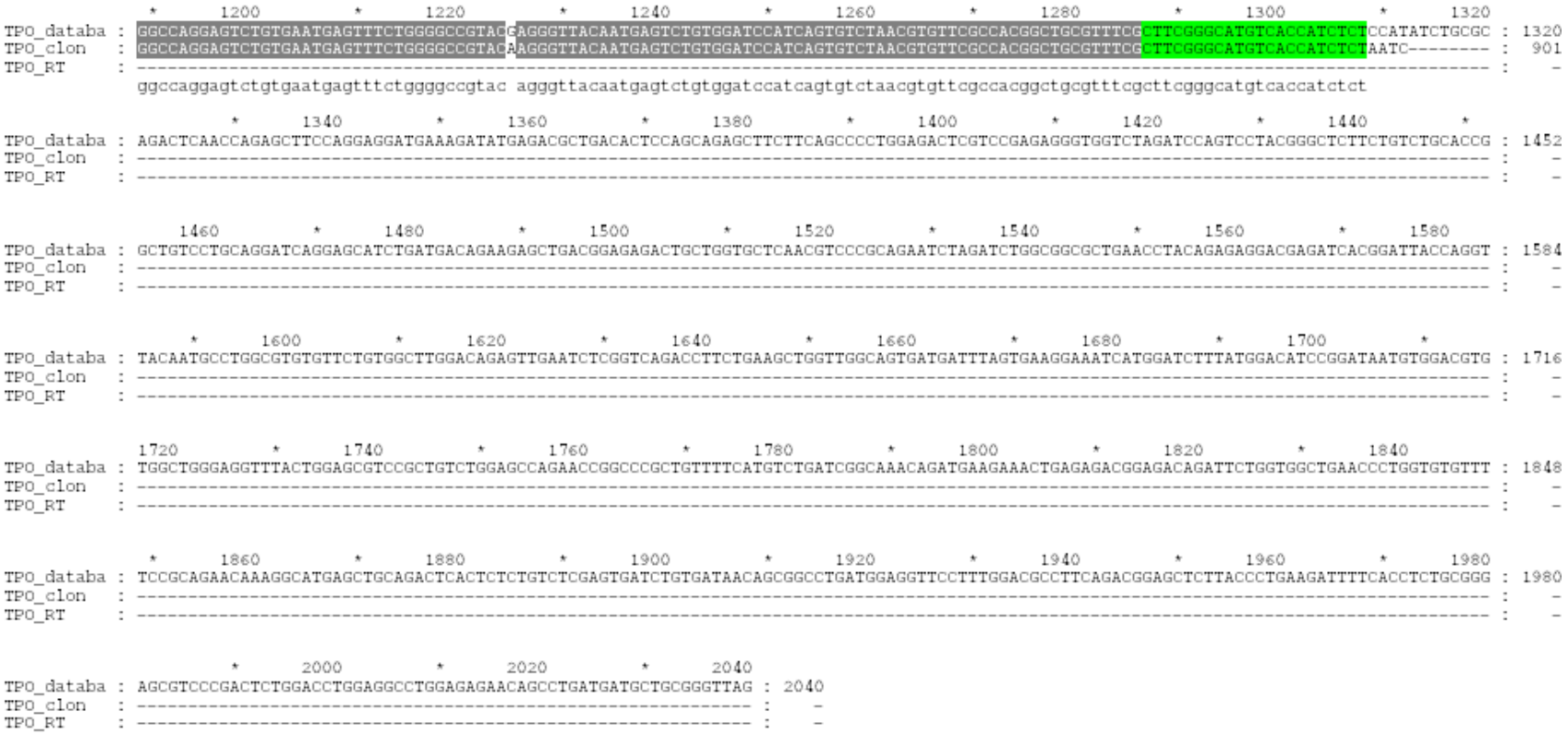


Figure A 5: TPO sequence alignments of the sequences from the database (TPO__datab), the sequence from the cloned fragment (TPO__clon) with the cloning primer sequence (green) and the qRT-PCR amplicon sequence (TPO_RT) with the primer sequence (yellow).

Annex 8: Tg alignment

Figure A 6: Tg sequence alignments of the sequences from the database (Tg__datab), the sequence from the cloned fragment (Tg__sequen) with the cloning primer sequence (green) and the qRT-PCR amplicon sequence (TPO_RT) with the primer sequence (yellow).

Annex 9: CtsBa alignment

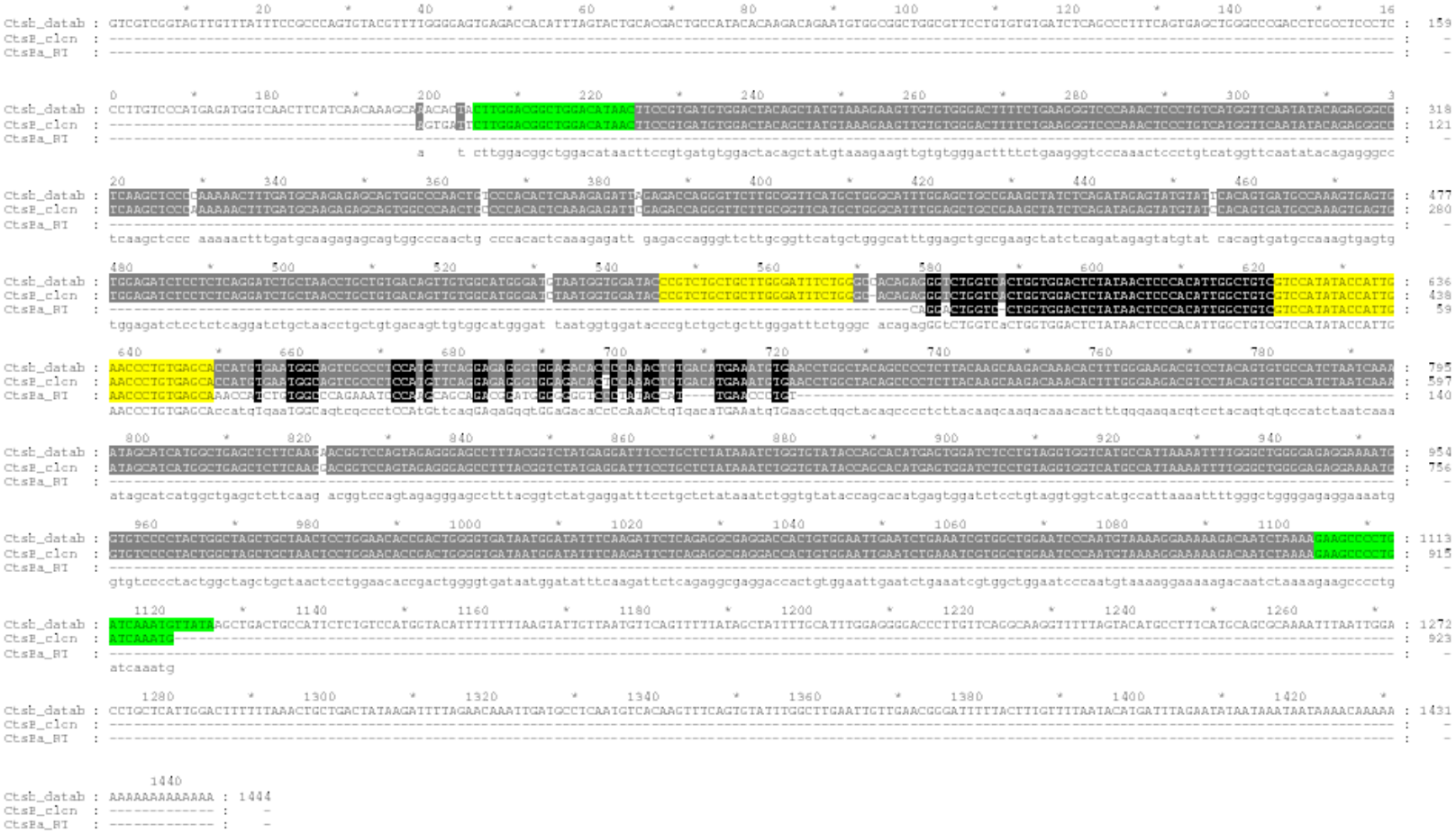


Figure A 7: CtsBa sequence alignments of the sequences from the database (CtsBa__datab), the sequence from the cloned fragment (CtsBa_clon) with the cloning primer sequence (green) and the qRT-PCR amplicon sequence (CtsBa_RT) with the primer sequence (yellow).

Annex 10: GADPH-2 alignment

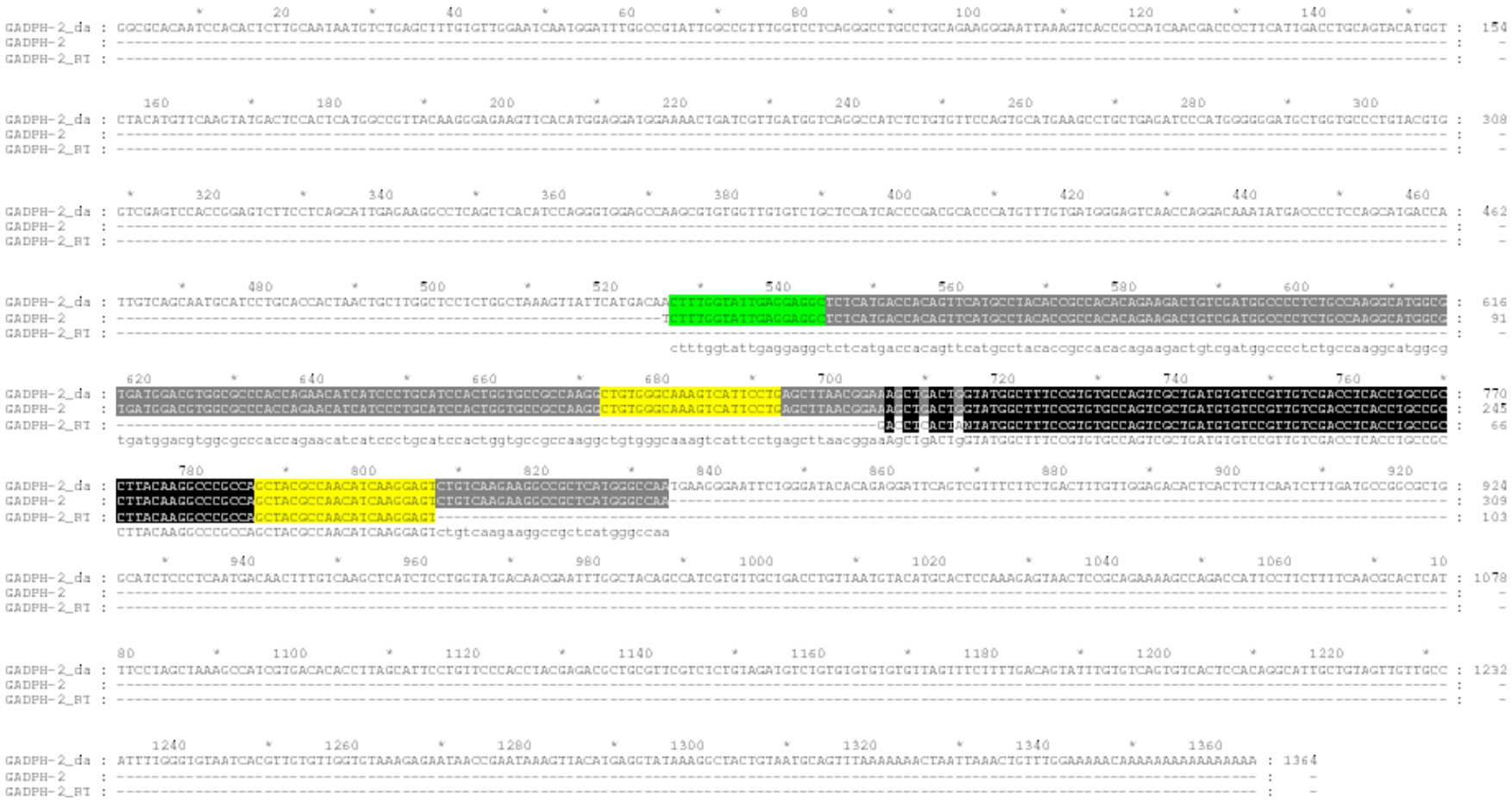


Figure A 8: GADPH-2 sequence alignments of the sequences from the database (GADPH-2_da), the sequence from the cloned fragment (GADPH-2) with the cloning primer sequence (green) and the qRT-PCR amplicon sequence (GADPH-2_RT) with the primer sequence (yellow).