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Thyroid active agents T3 and PTU differentially affect immune gene transcripts in the head kidney of rainbow trout (*Oncorynchus mykiss*)

Alba Quesada-García[a], Paloma Encinas[b], Ana Valdehita[a], Lisa Baumann[c], Helmut Segner[c], Julio M. Coll[b], José M. Navas[a,*]

[a] Instituto Nacional Investigaciones Agrarias y Alimentarias. INIA, Dpto. Medio Ambiente, Ctra. De la Coruña Km 7.5., E-28040 Madrid, Spain
[b] Instituto Nacional Investigaciones Agrarias y Alimentarias. INIA, Dpto. Biotecnologia, Ctra. De la Coruña Km 7.5., E-28040 Madrid, Spain
[c] Faculty of Vetsuisse, Centre for Fish and Wildlife Health, University of Bern, Länggasstra. 122, CH-3001 Bern, Switzerland

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ABSTRACT

In mammals, numerous reports describe an immunomodulating effect of thyroid-active compounds. In contrast, only few reports have been published on this subject in fish. We previously demonstrated that immune cells of rainbow trout (*Oncorhynchus mykiss*) possess thyroid hormone receptors (THRs) and that exposure of trout to the thyroid hormone 3,3′,5-triiodo-L-thyronine (T3) or the antithyroid drug propylthiouracil (PTU) alters immune cell transcript levels of THR and several immune genes. The present study aims to further characterize the immunomodulating action of thyroid-active compounds in trout immune cells. We report here the use of a custom-designed 60-mer oligo immune-targeted microarray for rainbow trout to analyze the gene expression profiles induced in the head kidney by T3 and PTU. Morphometric analyses of the thyroid showed that PTU exposure increased the size of the epithelial cells, whereas T3 induced no significant effects. Both T3 and PTU had diverse and partly contrasting effects on immune transcript profiles. The strongest differential effects of T3 and PTU on gene expressions were those targeting the Mitogen Associated Protein Kinase (MAPK), NFkB, Natural Killer (NK) and Toll-Like Receptor (TLR) pathways, a number of multipath genes (MPG) such as those encoding pleiotropic transcription factors (*atf1*, *junb*, *myc*), as well as important pro-inflammatory genes (*tnfa*, *tnf6*, *il1b*) and interferon-related genes *(ifng*, *irf10*). With these results we show for the first time in a fish species that the in vivo thyroidal status modulates a diversity of immune genes and pathways. This knowledge provides the basis to investigate both mechanisms and consequences of thyroid hormone- and thyroid disruptor-mediated immunomodulation for the immunocompetence of fish.

1. Introduction

In recent years, many chemicals ubiquitously present in the environment have been suspected of interfering with endocrine homeostasis. The so-called Endocrine Disruptors (EDs), are defined as "exogenous substances or mixtures that alter function(s) of the endocrine system and consequently cause adverse health effect in an intact organism, its progeny or (sub) populations" (WHO/IPCS. 2002). They are of particular concern since they are able to exert their deleterious effects at low concentrations (nanomolar range) and in some occasions long term effects appear after short exposures at particular labile periods, for instance during sexual development (Vandenberg et al., 2012). With respect to teleostean fish, most of the work related to endocrine disruption focused on the interactions of chemicals with the estrogen or androgen systems, probably due to the key role played by these hormones in reproduction and development. However, in recent years a number of studies have reported thyroidal activities in environmental samples demonstrating alterations in thyroid gland development and ratios of thyroid hormones in wild animal populations (Colborn, 2002). For instance, broiler and poultry manure applied as agricultural amendment show thyroidal activities that could end up in water courses after run-off or leakage (Valdehita et al., 2014). In fact, thyroidal activities have been reported in rivers and waste waters in France (Jugan et al., 2009). In addition, fish feed extracts were also able to activate thyroid receptor in stably transfected cells (Quesada-Garcia et al., 2012). All this evidences the necessity of studying the possible environmental effects of these activities, particularly on aquatic organisms including fish.

Thyroid hormones (THs) include L-thyroxine (T4) and the biologically active 3,3′,5-triiodo-L-thyronine (T3). T4 is secreted by thyroid follicles under the stimulation of the thyroid-stimulating

* Corresponding author. Fax: +34 913474008.
E-mail address: jmnavas@inia.es (J.M. Navas).

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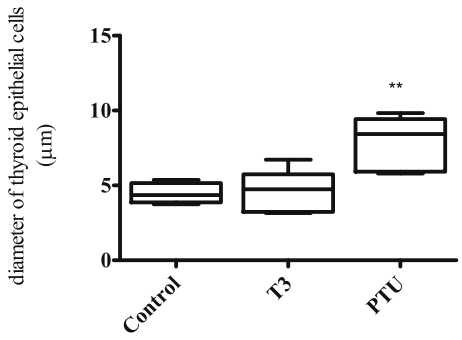

Fig. 1. Diameter of epithelial cells of thyroids from T3- and PTU-fed rainbow trout. Thyroid epithelial cells diameter measured in control, T3-fed and PTU-fed rainbow trout. Values represent mean ± SD ($n = 5$ trout). *, significantly different from control as $p < 0.05$ by the Student's t.

hormone (TSH) released by the pituitary. T4 is released to the blood and reaches target cells where it undergoes monodeiodination to be converted into T3. THs play a critical role in a wide variety of physiological functions such as development, metamorphosis and growth in all vertebrates including fish (Blanton and Specker, 2007). Therefore, most studies assessing the effect of thyroid-disrupting compounds have focused on such endpoints (Coimbra and Reis-Henriques, 2007; Crane et al., 2006; Sharma and Patino, 2013). Nevertheless, in mammals there is an increasing evidence of the interaction between the immune and the endocrine systems with complex bi-directional influences between both. For instance, in mammals, white blood cells and mast cells contain T3 (Csaba et al., 2004) and THs influence the distribution of lymphocyte subsets by affecting the differentiation of B lymphocytes, the proliferation of T lymphocytes, and the maturation of macrophages (De Vito et al., 2011, 2012; Perrotta et al., 2014). In addition, there is also an influence of the immune system on the thyroid system, for instance, several cytokines (i.e.,: il1, il6, tnfa) affect the release of pituitary hormones and the normal functioning of the hypothalamus–pituitary–thyroid (HPT) axis provoking negative effects on the thyroid functions (Haddad et al., 2002).

Much less is known on a possible immunomodulatory role of thyroid hormones in fish. There exists indirect evidence that they influence the immune system of fish. For instance, in hypothyroid fish, the numbers of circulating leukocytes were reduced (Slicher, 1961), and T4 or TSH administration altered lymphocyte numbers in hypophysectomized fish (Ball and Hawkins, 1976). Furthermore, T4 led to increased thymus size in developing zebrafish, suggesting a role of thyroid hormones in the development of this organ (Lam et al., 2005). Our groups recently demonstrated the presence of thyroid hormone receptors (THRs) in immune organs and isolated immune cells of rainbow trout (*Oncorhynchus mykiss*) (Quesada-Garcia et al., 2014). Remarkably, we found that transcript levels of *thra* (*thyroid hormone receptor alpha*) was clearly higher in immune organs and cells than in the liver, whereas *thrb* (*thyroid hormone receptor beta*) was predominant in the liver. In addition, exposure of rainbow trout to T3 or to the antithyroid agent, 6-propyl-2-thiouracil (PTU), led to a significant alteration in the relative expression of marker genes of immune cell subpopulations, an observation that points again to the immunomodulatory activity of the thyroid system in fish (Quesada-Garcia et al., 2014).

In recent years the advances in genomics have favored the emergence of high throughput tools such as expression microarrays which have substantially contributed to the increased understanding on the molecular modes of action of endocrine substances as well as to the characterization of the involved signaling pathways. In particular, custom-made microarrays offer an important advantage, as they enable a design enriched for genes or pathways of interest, focusing the study towards a particular objective. For instance, custom microarrays enriched for hormone-responsive genes were used to assess the transcriptional effects of endocrine-active compounds on common carp (Moens et al., 2006) or of cortisol on LPS-activated monocytes from rainbow trout (MacKenzie et al., 2006). These tools are also of value in unraveling the toxic modes of transcriptional action of environmental contaminants, including Endocrine Disruptors. In fact, in ecotoxicology an own subdiscipline called ecotoxicogenomics has emerged which is devoted to "the study of gene and protein expression in wild animal species that are important in responses to environmental toxicant exposures" (Iguchi et al., 2006, 2007; MacKenzie et al., 2006; Miracle and Ankley, 2005; Moens et al., 2006; Snape et al., 2004; Villeneuve et al., 2012).

In the present study, we used a custom 60-mer oligo microarray, consisting of immune-related gene sequences (immune-targeted microarray) from rainbow trout to analyze gene expression profiles induced by thyroid-active compounds in the head kidney of juvenile rainbow trout. The test agents used in this study were the thyroid hormone 3,3′,5-triiodo-L-thyronine (T3), and the antithyroid drug propylthiouracil (PTU). The results of the microarray were validated by reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) (Ballesteros et al., 2012; Chinchilla et al., 2015). The objective of this work was to explore molecular pathways, particularly those related with the immune system, which were altered in rainbow trout after treatment with T3 or PTU. This will provide the basis for better understanding the interaction between the thyroid and the immune systems, and how environmentally induced changes in the immune system may translate into altered immune function and immunocompetence. Importantly, immunomodulating effects of endocrine-active compounds may not result in overt alterations of the resting immune system, but may become evident only after activation of the immune system by pathogen challenge (e.g. (Kollner et al., 2002; Wenger et al., 2011), a phenomenon which may be designated as "silent endocrine disruption". Taking all this into account, this work has concentrated on the description of genes, particularly those related with immune system, that were altered in trout after treatment with T3 or PTU. It intends to serve as a basis for further studies going more deeply on the effects of pollutants acting as agonists or antagonists of thyroid hormones. Irrespectively of their use as model compounds, the substances used in this work could potentially appear in waters contributing to pollution and causing toxicity for aquatic organisms. For instance, the presence of thyroid hormones has been reported in Finnish water bodies (Svanfelt et al., 2010) and the production and use of PTU as therapeutic thyroid inhibitor can lead to its release to the environment through waste water (NLM, 2004).

2. Materials and methods

2.1. Chemicals

All chemicals were purchased from Sigma–Aldrich, (Madrid, Spain) unless otherwise noted.

2.2. Handling of rainbow trout

Juvenile rainbow trout (49 ± 2.7 g body weight) were selected from a stock population reared in an outdoor facility and were transferred to indoor aquaria (200 l) with flow-through water supply (0.5 l/min) in air-saturated tap water ($CaCO_3$ 2.13 ppm, chlorine dioxide < 0.01 mg/l) and at a water temperature of 15 ± 1 °C and artificial light (12:12 h). The fish were checked for infectious pathogens prior to the start of the experiment. Water parameters were as follows (Oxygen: 8.0–8.5 mg/ml; pH: 7.3–7.4; carbonate

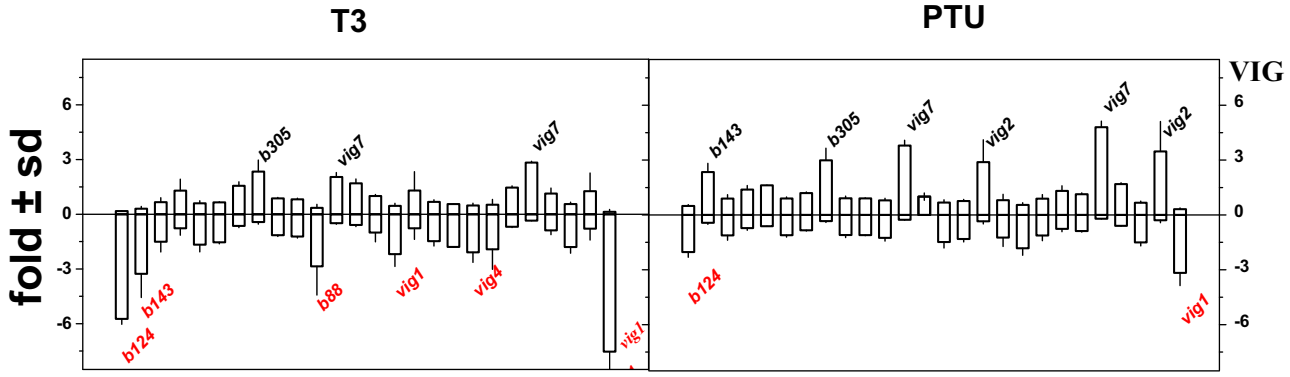

Fig. 2. Hybridization of T3- or PTU-fed trout transcripts to immune-targeted microarray. Differential transcript expression of VHSV-inducible genes (VIG). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
Trout were fed with T3 or PTU, RNA was extracted from the head kidney 7-days later, labeled with Cy3 and hybridized to the microarrays. After normalization and outlier removal, the mean and standard deviations were represented ($n = 4$). Gene symbols with differential expression fold change >2 or <2 are nearby the corresponding bars. Fold change were calculated by the formula, fluorescence of T3- or PTU-fed trout per probe/fluorescence of control trout per probe. **+**, upregulated fold change in black. –, downregulated fold change in red.

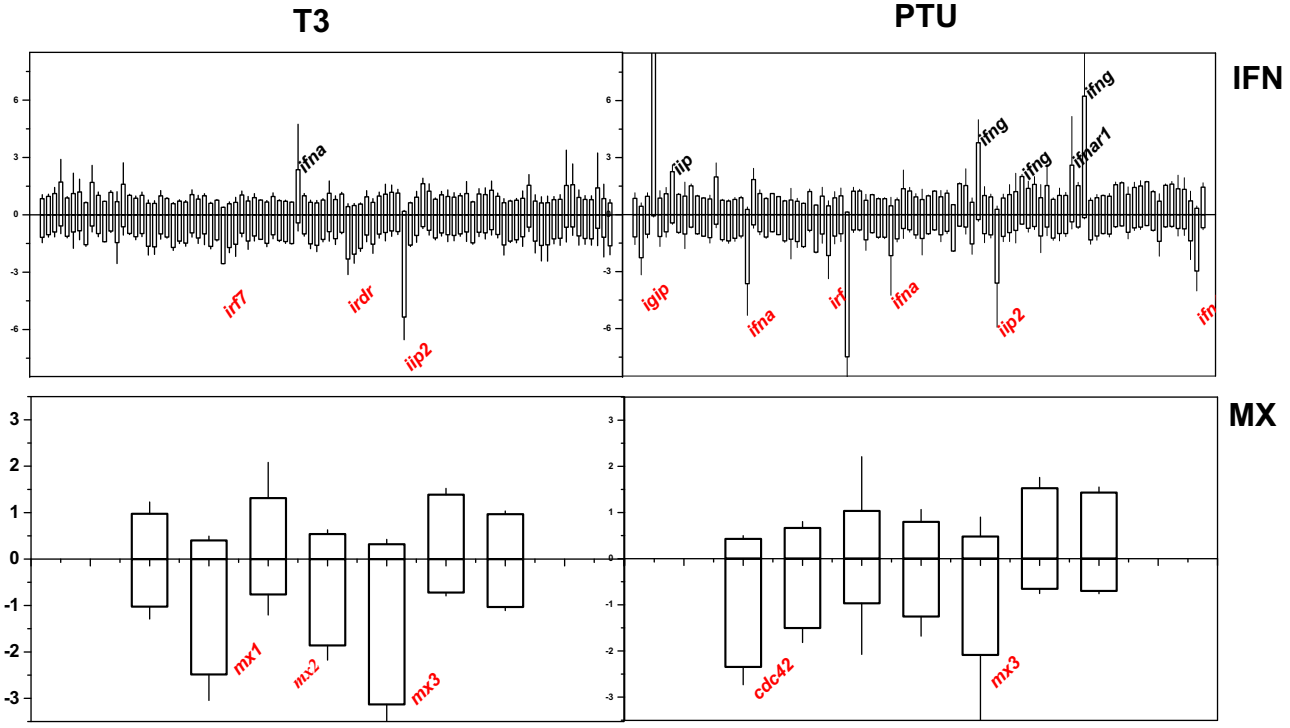

Fig. 3. Hybridization of T3- or PTU-fed trout transcripts to immune-targeted microarray. Differential transcript expression of *interferon* (*IFN*) and *myxovirus-induced proteins* (*MX*).
Legend as described for Fig. 2.

hardness: 13–14; Ammonium: 0 mg/L; Nitrite: 0.05 mg/L; Nitrate: 10–25 mg/L).

Each of the 3 rainbow trout groups were fed daily with 1% of the body weight of fish with a commercial diet spiked either with 20 μg of T3 per g of feed (group I), or with 5000 μg of the anti-thyroid agent 6-propyl-2-thiouracil (PTU) per g of feed (group II). T3 and PTU were dissolved in ethanol and sprayed over the feed pellets, which were then dried at 60 °C to allow for solvent evaporation. The control group III was fed pellets that were sprayed only with ethanol. The T3/PTU concentrations were selected by following data from the literature (For T3: Finnson and Eales, 1999; Takagi et al., 1994 working on rainbow trout. For PTU: Peter and Peter, 2009 working on tilapia, *Oreochromis mossambicus*)

Seven days after the start of the treatments, fish were sacrificed by immersion in a solution of 100 mg/l of tricaine methane sulphonate (MS-222, Argent Chemical Laboratories, Redmont, CA, USA) in water. Head kidney was immediately extracted and those from every 2 fish were pooled and stored in RNA-later (Sigma–Aldrich) until RNA extraction. All procedures were performed following the National and European Commission guidelines and regulations on laboratory animal care. All efforts were made to minimize animal suffering.

2.3. Histological preparations

Heads from 5 trout per treatment were fixed in 10% buffered formalin for 24 h and decalcified (Decalcifier, rapid, J.T. Baker)

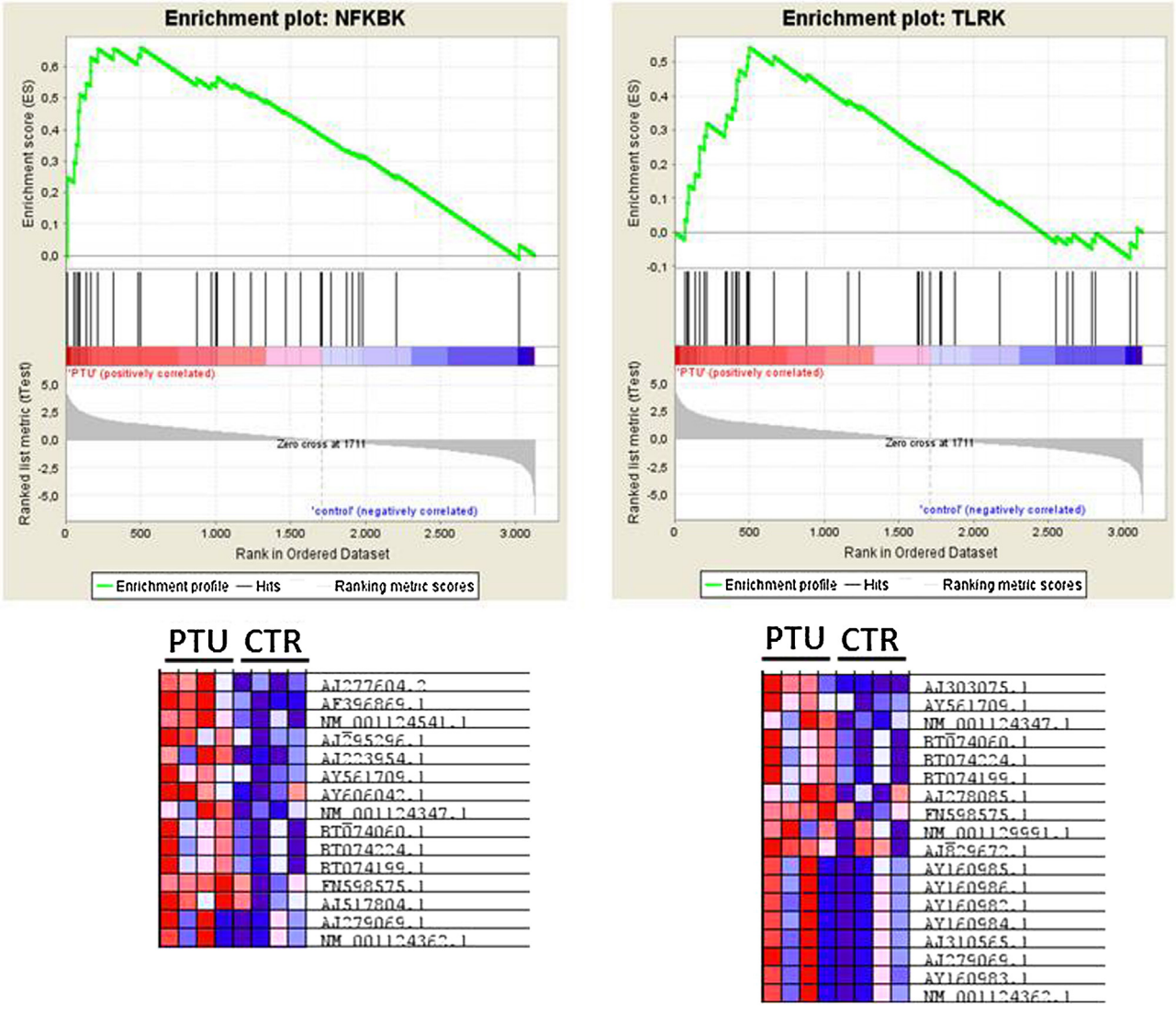

Fig. 4. Enrichment plots for NFkB and TLR pathways in PTU-fed rainbow trout. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
Enrichment plots for NFkB and TLR KEGG (K) pathways (up) and their corresponding heat maps (down) for the leading edge genes. The enrichment scores per gene for each pathway are plotted against all the genes present in the microarray ordered by their expression metrics. **Red**, genes correlating with the PTU-fed phenotype (PTU). **Blue**, genes correlating with the control phenotype (CTR). Each square of the heat maps correspond to one biological replicate.

Table 1
List of modulated genes which were present in >6 immune-related pathways (mMPG) ranked by the number of pathways.

Symbol	Accession Number	Description of the gene product	n° Paths	T3-C Mean	T3-C ±sd	PTU-C mean	PTU-C ±sd	*n*
#	NM_001124559.1	cAMP-dependent transcription factor	19	***0.50**	0.12	***0.56**	0.05	4
tnf6	TC121157	Tumor necrosis factor lig superfamily6	13	1.27	0.28	***1.56**	0.01	4
tnfa	AJ277604.2	Tumour necrosis factor alpha	12	1.27	0.58	***2.37**	0.28	16
ifng	NM_001124620.1	Interferon gamma	11	1.16	0.10	***1.67**	0.28	16
lck	NM_001124541.1	LCKinase 1	11	0.86	0.02	***1.71**	0.16	16
mhc2.daa	AJ251431.1	MHC class II alpha (onmy-DAA)	10	1.63	0.61	***2.81**	0.81	12
#	NM_001124520.1	JunB transcription factor	10	***0.62**	0.06	0.86	0.15	4
#	NM_001124699.1	v-myc myelocytomatosis viral oncogen	9	1.12	0.23	***0.34**	0.05	8
il1b	NM_001124347.1	Interleukin-1-beta	8	1.33	1.20	***2.92**	0.76	8
irf10	AJ829672.1	Interferon regulatory factor 10	8	0.79	0.16	***1.73**	0.03	4
il8	AY160982.1	Interleukin-8	7	***2.05**	0.32	***1.62**	0.21	44
nfkbia	BT074224.1	NF-kappa-B inhibitor alpha	6	1.13	0.36	1.49	0.16	12

Differentially expressed genes with folds >1.5 (upregulated) or <0.66 (downregulated) present in >6 pathways (modulated multipath genes, mMPG) together with their corresponding mean folds were filtered/extracted from the microarray data. The mMPG were tabulated together with their corresponding mean folds and standard deviations by comparing T3 or PTU with control trout. The genes were then ranked by the number of pathways in which they were present. Other genes common to >6 pathways were not differentially expressed; for instance: *p53* (NM_001124692.1, common to 16 pathways), *il6* (NM_001124657.1, 11), *if2a* (NM_001124296.1, 9), *tgfb* (X99303.1, 9), *il12b* (AJ548830.1, 8), *grb* (AY173044.1, 7), *irak4* (FN598575.1, 6), etc.

[#] pleiotropic transcription factors.

Table 2
Immune-related pathways containing the highest number of mMPG and percentage of modulated genes per pathway.

Immune-related pathway	Data base	mMPG	Total n° of modulated genes	Total n° genes/pathway	% modulated
MAPK	K	6	6	17	35.3
Natural Killer (NK)	K	5	8	10	80.0
Influenza	K	5	9	15	60.0
Toll-Like Receptor	K	4	10	24	41.6
NFkB	K	4	12	23	52.2
MAPK signaling	W	4	4	11	36.4
JAK-STAT	K	4	4	10	40.0
Cytokines inflammatory response	W	4	8	16	50.0
Antigen processing	K	4	5	12	41.7

To estimate the most targeted pathways by either T3 or PTU, the number of genes having differential expression folds <0.66 or >1.5 (modulated) in T3-C or PTU-C data in each of the immune-related pathways were calculated (mMPG from Table 1). Only the data from pathways having more than 10 genes in the microarray and >3 mMPG (Table 1) were tabulated. None of the studied immune-related pathways have >3 mMPG in the T3-fed trout.The MAPK (Mitogen Associated Protein Kinases) were identified in both KEGG (K) and WIKI (W) pathway data bases.The percentage of modulated genes were calculated by the formula, 100 × number of total modulated genes per pathway/total number of genes per pathway.

Table 3
Up (positive NES) and down (negative NES) regulated gene sets in PTU and T3-fed rainbow trout by the GSEA method.

Treatment	Pathway ID[a]	Pathway description	Size	NES
T3	map04064	NFkB	33	**1.91
	map04620	TLR	37	**1.69
	wp170	Nuclear receptors	11	*−1.49
	map05168	Herpes simplex infection	36	*−1.50
	wp231	TNF signaling pathway	17	*−1.55
	wp1348	Androgen receptor signaling	31	*−1.58
	wp1835	Interferon alfa/beta signaling	12	**−1.75
	wp585	Interferon type I signaling	15	**−1.88
PTU	map04064	NFkB	33	**2.15
	map04650	Natural Killer (NK)	13	**2.12
	map04620	TLR	37	**1.86
	map05320	Autoimmune thyroid disease	14	**1.84
	map04666	Fc gamma R-mediated phagocytosis	17	*1.84
	map04622	RIG-I-like receptor signaling pathway	18	**1.78
	map04062	Chemokine signaling pathway	12	**1.77
	wp530	Cytokines and inflammatory response	17	**1.66
	map04144	Endocytosis	11	**1.58
	map04621	NOD-like receptor signaling pathway	19	**1.50
	map05164	Influenza A	17	**1.49
	map04610	Complement and coagulation cascades	15	**−1.99

NES, Normalized Enrichment Scores from the GSEA method. Pathways ordered from higher to lower NES in each T3- and PTU-fed lists. Size, number of genes per pathway.
**Bold, FDR $q < 0.05$.
*FDR $q < 0.25$.
[a] Pathways named "map" belong to KEGG database while those named "wp" belong to the Wikipathways database. Pathways belong to *Homo sapiens*, except wp1348 which belongs to zebrafish.

for 45 min before automated processing in an ascending series of ethanol, followed by xylol and final embedding in paraffin (Histosec, Merck). Median sections of 3 μm thickness were made from the thyroid follicles in the mouth/gill region of each fish. Sections were stained with hematoxylin and eosin (HE). Morphometric analyses were performed with a light microscope (Olympus BX51) with associated camera (ProgRes C5) and software (ProgRes CapturePro, v2.8.8) at 100× magnification. For each individual trout, the slide was chosen that contained the maximal number of thyroid follicles. The diameter of thyroid epithelial cells from 10 representative follicles was measured at 5 different positions around each side of the follicle. This resulted in 50 measurements for each individual trout and 250 for each treatment (T3, PTU, control). The means and their standard deviations for each thyroid epithelial cell diameter were calculated for each treatment.

2.4. RNA extraction

The head kidney from each of the 2 pooled trout were homogenized using the Tissue Lyser Cell Disruptor (Qiagen S.A., Spain) for 10 min at 50 Hz with 3 mm glass beads in lysis RLT buffer (Qiagen S.A.). Their RNA was then extracted from the homogenates by the RNAeasyPlus kit (Qiagen S.A.) and eluted in RNase-free water. RNA concentrations were measured by Nanodrop (ThermoScientific, DE, USA) and the presence of 18 and 28 S bands confirmed by denaturing RNA agar electrophoresis (Sigma–Aldrich). Additional RNA quality control (RNA integrity number, RIN) was performed by Nimgenetics (Madrid, Spain). For each trout group (T3, PTU, control), 4 of the best quality RNA (RIN > 8.0) samples were chosen for microarray hybridization.

2.5. Design of rainbow trout immune-targeted microarrays

The microarrays were enriched with rainbow trout immune-related genes (immune-targeted microarrays) as described previously (Ballesteros et al., 2012; Chinchilla et al., 2015). The final 8 × 15 K microarray corresponds to Agilent's ID032303 (Gene Expression Omnibus GEO platform submission number GPL14155), that contains 60-mer annotated immune-related probes each by duplicate. To simplify the analysis of results, annotated probes were classified according to several gene groups: viral hemorrhagic septicemia virus (VHSV)-inducible genes (VIG) (number of probes, $n = 26$); genes encoding IFN, interferons and their receptors ($n = 92$); genes encoding MX, interferon-inducible proteins mx ($n = 8$); genes encoding TLR, Toll-Like Receptors ($n = 32$); genes encoding CO, com-

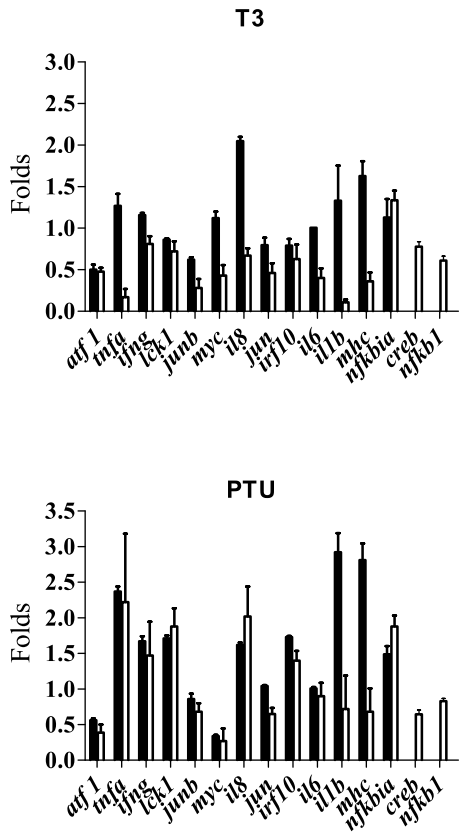

Fig. 5. Comparison between differential expression fold change of selected transcripts obtained by hybridization to microarray (black bars) and RT-qPCR (white bars).
The RNA samples were the same than those used for the microarray. The cDNA were obtained, and RT-qPCR performed as indicated in methods by using SYBR green. The elongation factor 1α (*eef1a*) gene was used to normalize the results. All qPCR reactions were performed in triplicate and the results were expressed as mean ± standard deviation. The expression levels were calculated as relative fold change of the expression of *ef1a* according to the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). **Black bars**, data from the microarray. **Open bars**, data from the RT-qPCR.

plement components ($n = 177$); genes encoding IL, interleukins and its receptors ($n = 120$); MA, macrophage related genes ($n = 126$); genes encoding TNF, tumor necrosis factor ($n = 33$); genes encoding CD, cluster differentiation antigens ($n = 59$); genes encoding CK, chemokines and their receptors ($n = 122$) and genes encoding TF, transcription factors ($n = 672$). Other probes included in the microarray belong to genes encoding immunoglobulins and T-cell receptors. Although included in the microarray, they were not analyzed here due to the high number of sequence variations that were contained. The trout microarray ID32303 used for these experiments was previously validated by RT-qPCR (Ballesteros et al., 2012; Chinchilla et al., 2015).

2.6. Hybridization of trout transcripts to the immune-targeted microarrays

Labeling of 2 μg of RNA (50 μg/ml) and hybridization to the microarrays were performed by Nimgenetics (Madrid, Spain) by complying with the Minimum Information About a Microarray Experiment (MIAME) standards. The gProcessedSignal was chosen for statistical analysis after filtration of the data and the two technical replicates per probe per microarray were averaged as described previously (Ballesteros et al., 2012; Chinchilla et al., 2015).

2.7. Statistical analysis of microarray data

Individual fluorescence values of each probe were normalized relative to all probe fluorescences for each microarray according to the formula, fluorescence of each probe/sum of all the probe fluorescences per microarray. The raw and normalized data were deposited in GEO (number GSE54422). After normalization, outlier values (defined by those fluorescence values > or < mean ± standard deviation, $n = 4$) were identified and eliminated (masked) from the calculations programmed in Origin pro 8.6 (OriginLab Corporation, Northampton, USA). Differential expressions were calculated in fold change for each probe replica as reported previously (Ballesteros et al., 2012; Chinchilla et al., 2015) by using the following formula: fluorescent value from T3 or PTU-fed trout/mean fluorescent value from control trout. Means and standard deviations of the fold change were then calculated (with a maximal of $n = 4$, depending on the number of removed outliers). The two-tailed independent Student-t statistic-associated p-value was also computed for each probe. A double simultaneous criterion to define differentially expressed gene transcripts was used: (**i)** genes with fold change >2 or <0.5 (to draw figures) and >1.5 or <0.66 (to search for multipath genes) and (**ii)** fold change deviated from the null hypothesis at $p < 0.05$.

2.8. Classification of rainbow trout genes in immune pathways and identification of genes common to several pathways (multipath genes)

To classify the rainbow trout genes of the ID032303 immune-targeted microarray into immune pathways, the Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genome.ad.jp/kegg/) and the WikiPathways (WIKI) (http://wikipathways.org/index.php/WikiPathways) pathway databases corresponding to human pathways were used. Rainbow trout genes orthologous to human genes from 74 immune-related pathways were manually searched on GenBank sequences (accessed from February to June of 2013). The list of the rainbow trout genes classified by pathways and their accession numbers were deposited in the GEO platform with submission number GPL17758. Each of the probes and their corresponding data from the ID032303 microarray were then classified into the above mentioned rainbow trout pathways by using a macro in Excel. Multipath genes defined as the genes present in at least 6 pathways (Encinas et al., 2013) were then extracted by using a program made in Origin. Those multipath genes showing significant differential expression fold change >1.5 or <0.66 at the $p < 0.05$ level were then tabulated.

2.9. Gene Set Enrichment Analysis (GSEA)

The Gene Set Enrichment Analysis (GSEA) methodology (Subramanian et al., 2007; Subramanian et al., 2005) was used to evaluate the microarray data at the level of different Gene Sets (GS). The list of gene values were ranked by using the GSEA *t*-test statistic metric (Subramanian et al., 2007; Subramanian et al., 2005) obtained by comparing the following rainbow trout phenotypes: T3-fed versus control and PTU-fed versus control. We selected the initial gene sets (GS) defined for the pathways of the microarray (see above). Each of the genes from each Gene Set was then classified according to the GSEA Enrichment Score (ES) and compared to each other by using the Normalized ES (NES) which corrects for number of genes. For assessing NES significance, the False Discovery Rate (FDR) was used after comparing with the corresponding null distribution obtained after averaging 1000 GS permutations (1000 random gene combinations per GS). As suggested by GSEA

the most stringent cut-off value of FDR $q < 0.05$ was generally used for NES significance.

2.10. Quantitative estimation of transcripts for selected immune-related genes by RT-qPCR

Results from the microarray analyses were validated by RT-qPCR using the primers listed in Table S1. The selection of the genes to be validated by RT-qPCR was not random, but on the contrary we selected relevant, multipath genes (present in ≥ 6 immune-related pathways). In addition, we selected two genes which were not present in the array (*creb* and *nfkb1*) but that play key roles in several pathways and whose interaction with the thyroid signaling pathway has been previously suggested in mammals (Gupta et al., 2013). Their sequences were found among rainbow trout EST (CA341859 and BX887809, respectively). The low *E*-values of the matches obtained when performing BlastX of those EST against the NCBI database confirmed that they belong to *nfkb1* and *creb*, respectively.

Reverse transcription (RT) of the RNA was carried out in 20 μL of reaction volume containing 500 ng Random Primers, 2.5 mM $MgCl_2$, 2 mM dNTPs, 20 U RNAse inhibitor, 160 U M-MLV reverse transcriptase (Promega AG, Wallisellen, Switzerland) and 500 ng total RNA. The RT-qPCR were then performed by using SYBR green, in a Line-Gene 9600 System (BIOER Technology, Hangzhou, China). Each reaction mix contained 1X HotSybr RealTime PCR MasterMix1X (MCLAB, San Franciso, CA, USA), 300 nM of each primer, and 1 μL of cDNA. The thermal profile was 10 min at 95 °C, followed by 40 amplification cycles of 5s at 95 °C and 30 s at 60 °C. After the run, a dissociation curve analysis (heating from 56 °C to 95 °C) was performed in order to verify the amplification of the correct product. Relative quantification was performed using the comparative Ct (cycle threshold, defined as the number of cycles required for the fluorescent signal to cross the threshold, background level) method, also referred to as $2^{-\Delta\Delta CT}$ (Livak and Schmittgen, 2001), representing the amount of target gene normalized to the endogenous control gene (*elongation factor 1 α*, *ef1a*) and relative to the mean value of the control group. That is, $\Delta\Delta Ct = (Ct\ target - Ct\ ef1a)_{treated\ groups} - (Ct\ target - Ct\ ef1a)_{control\ group}$. Given the constant expression of *ef1a* among tissues and treatment groups, which was checked in a preliminary study (Quesada-Garcia et al., 2014) (data not shown), it was selected as housekeeping gene.

3. Results and discussion

The effectiveness of the applied treatments to provoke changes in the thyroid axis was demonstrated by measuring by qPCR modifications in the expression of genes related with the thyroid system. These results have been previously published (Quesada-Garcia et al., 2014) and are not presented here, but we must mention that we observed changes in the expression of *thra* and *thrb* in immune cells and of *deiodinase 2* (*dio2*) in liver. In the case of *dio2* fish exposed to T3 showed lower expression compared to control while PTU-exposed fish showed the opposite pattern (Quesada-Garcia et al., 2014), which is consistent with the results obtained by other (Johnson and Lema, 2011; Mol et al., 1999; Noyes et al., 2013). Since the hepatic *dio2* is a biomarker commonly used to monitor thyroid status changes (Mol et al., 1999; Noyes et al., 2013), these results indicate that the treatments were effective in provoking alterations in the trout's thyroid axis.

3.1. Histological changes in thyroid tissues after treatment with T3 and PTU

In order to evaluate whether the T3 and PTU treatments affected thyroid, a morphometric analysis of epithelial height of the thyroid follicles was done. The average diameter of the follicular epithelial cells was 4.48 ± 0.30 μm ($n = 5$ trout) in control fish, 7.83 ± 0.81 μm ($n = 5$) in PTU-fed trout, and 4.54 ± 0.64 μm ($n = 5$) in T3-fed trout. While the difference between control and the PTU treatment was significant, follicular epithelial heights of T3-fed fish did not differ from the controls, but they displayed higher variability (6.6, 10.3 and 14% coefficient of variation, for control, T3- and PTU-fed trout, respectively) (Fig. 1).

The epithelial cells of the thyroid follicles are responsible for the production of THs and their release into the blood stream (Blanton and Specker, 2007). Therefore, changes in their cell size represent a meaningful indicator of thyroid activity. PTU inhibits the synthesis of THs by competitive binding to thyroid peroxidase (TPO), an essential enzyme for TH synthesis. This results in lowered TH levels, which in turn stimulates increased TSH release from the pituitary, and consequently leads to the observed hyperthrophy of the epithelial follicle cells attempting to compensate for the PTU-induced decrease of TH production. A compensatory hypertrophy of thyroid follicle cells under PTU treatment has been observed in other fish species as well: Schmidt and Braunbeck (2011), when treating developing zebrafish with different concentrations of PTU, found thyroid follicle hyperplasia and hypertrophy. Similar observations were made by Van der Ven et al. (2006). Also other thyroid disrupting chemicals with such a mode of action are known to cause thyroid follicle hypertrophy (Palace et al., 2008; Park et al., 2011). The T3 treatment did not cause significant hypo-trophy of the epithelial cells, as it might be expected, indicating that the fish were able to deal with the dietary TH supplementation within the boundaries of their physiological homeostasis.

3.2. Overview of head kidney probes/genes identified by hybridization to rainbow trout immune-targeted microarrays after being fed with T3 or PTU

The distribution of signal intensities shows a higher number of probes down regulated more than one fold change in PTU- than in T3-fed trout, suggesting an overall stronger immunosuppressive effect of PTU (Fig. S1).

A more detailed analysis of the differential expression of transcripts was then undertaken by using gene groups (not shown) (see Supplementary Tables S2 and S3 for an extensive comparison of the genes which were significantly up (S2) or downregulated (S3) in head kidneys of T3 or PTU-fed rainbow trout). Most variations were found for the VHSV-inducible genes (*VIG*) and interferon-related gene groups (*IFN* and *MX*). *VIG* represent a group of immune genes that were identified in *in vitro* studies with leukocytes isolated from rainbow trout head kidney to be responsive to challenge by the viral hemorrhagic septicemia virus (VHSV) (O'Farrell et al., 2002) (Fig. 2). T3 significantly downregulated 6 *VIG* probes and PTU significantly upregulated 6 *VIG* probes. Both T3 and PTU downregulated *vig1* (Boudinot et al., 1999) and *b124* and upregulated *vig7* (two probes) and *b305*. Contrasting effects of the two agents were observed for *b143*, which was downregulated by T3 but upregulated by PTU. Most functions of *VIG* are still unknown (Boudinot et al., 1999, 2000, 2001), but the results from this study provide the first evidence that these pathogen-responsive genes are regulated by the thyroid hormone status in vivo, having neither uniformly inducing nor suppressing effects.

Interferon-related genes (Fig. 3, *IFN*) showed important variations under T3 or PTU treatment. Again, there existed no uniform but a rather diverse response of the immune genes to the test agents: T3 significantly upregulated only one gene, *ifna* (*type I interferon*), while it downregulated *irf7*, *irdr* and *iip2*. PTU increased transcript levels of *ifng* (*type II interferon*) and *ifnar1*, while it downregulated a number of genes encoding interferon-related products including *ifna*, a gene that was upregulated by T3 was

downregulated by PTU. A more uniform response was observed for the *myxovirus-inducible interferon-related* genes (*mx* isoforms) (Trobridge et al., 1997; Trobridge and Leong, 1995), for which both T3 and PTU had mostly a downregulating activity (Fig. 3, MX). Downregulation of *mx* isoforms has been also observed in response to recombinant non-virion (rNV) protein (Chinchilla et al., 2015) as a mechanism of VHSV-induced immunosuppression to favour viral replication. Therefore, the downregulation of *mx* isoforms by T3/PTU might compromise the defence capacity of trout towards infectious viruses.

Both T3 and PTU influenced transcript levels of immune-related genes. A pronounced upregulating effect of T3 was evident for genes of the complement pathways, including *c7*, *c8* and *complement factor bf2*. The gene encoding a lectin pathway component, c-type mbl-2 (Nikolakopoulou and Zarkadis, 2006) was also upregulated. With respect to genes encoding chemokines, T3 was not very active (upregulation of the chemokine encoding genes, *ck7a, cxx10 g, vicxc,* and the pro-inflammatory interleukin encoding genes *il8* and *il6*). In contrast, PTU induced upregulation of 11 transcripts of genes encoding chemokines and interleukins as well as their receptors (*il1b il6, il8*) as well as genes encoding four antimicrobial peptides.

The experimental modulation of the thyroid status by T3 or PTU exposure modulated also the transcript levels of genes related to the innate and macrophage systems. Among the gene probes classified as macrophage-related *pentraxin* (the product of which is an acute phase response protein similar to the C-reactive protein) was downregulated >20-fold change in PTU-fed trout. *Pentraxins* are key components of innate immunity conserved from insects to humans and their products play a central role in antimicrobial resistance by binding to a number of pathogens and triggering complement-dependent cell lysis of infectious as well as infected cells (Bottazzi et al., 2010). They have been isolated in a number of piscine species including rainbow trout, as well as Atlantic salmon, cod (*Gadus morhua*) and snapper (*Pagrus auratus*), among others (Cook et al., 2003; Lund and Olafsen, 1998). Several reports have demonstrated an increase of pentraxin levels following bacterial lipopolysaccharide exposures (Cook et al., 2003) or DNA vaccination (Ballesteros et al., 2012). However, a few studies have shown downregulation (Hook et al., 2006; Olsvik et al., 2011). For instance, Hook et al. (2006) reported a 6-fold downregulation in rainbow trout *pentraxin* after exposure to the endocrine-disrupting compound, ethinylestradiol (EE2). Further immune genes prominently involved in antibacterial responses and downregulated by PTU include the *antimicrobial peptides cathelicidin*, *liver-expressed antimicrobial peptide 2B*, β-*defensin 1* and β-*defensin 3* (this last transcript showing >14-fold change downregulation). Similarly to *pentraxin*, piscine β-*defensin* genes are usually upregulated during infections (Casadei et al., 2009; Ruangsri et al., 2013) providing immunity not only against bacterial pathogens but also to viruses (Falco et al., 2008).

3.3. Identification of modulated genes common to several pathways (MultiPath Genes, MPG) suggests functionally important T3 or PTU targets

In a next step, we analyzed those differentially expressed genes that are common to several rainbow trout/human orthologous immune pathways (multipath genes, MPG). Also the fact that a rather high number of immune-related genes was regulated after T3 or PTU feeding suggested to prefer a targeted pleiotropic key gene analysis over the gene-by-gene analysis. Twelve mMPG fulfilling the multipath gene criteria (see Section 2) were identified (Table 1) as well as the pathways with the highest numbers of mMPG (Table 2). Treatment with T3 or PTU downregulated *atf1* (*cAMP-dependent transcription factor*) and T3 also downregulated the related *junb* (*jun proto-oncogen*). Both transcription factors are present in 19 or 10 immune-pathways, respectively, and they are part of some of the pathways with the highest numbers of mMPG (Table 2), for instance, MAPK (in both KEGG and WIKI pathways), JAK-STAT and Antigen Processing (not shown). All remaining mMPG (*tnf6, tnfa, ifng, lck, mhc2.daa, il1b, irf10, il8)* were upregulated by PTU, while only *il8* was upregulated by T3 (Table 1). The multipath analysis not only revealed *atf1/junb* to be one the most prominently regulated gene target in the T3 or PTU treatment action, but also showed that other mMPGs were upstream or related to *atf1/junb,* for instance, *tnf6* and *tnfa*. The *atf1/junb* belongs to genes encoding transcription factors participating in the formation of AP-1 (activating protein 1) heterodimer which targets TPA (Tumor Promoter Antigen) transcription binding factor sites on the DNA and has many immunological functions (Manicassamy and Pulendran, 2009; Parker et al., 2007; Schroder et al., 2006; Schroder and Bowie, 2007; Takeuchi and Akira, 2007; Thompson and Iwasaki, 2008). Therefore, the finding of this study that both PTU and T3 significantly downregulated *atf1/junb* expression which plays a key role in several immune pathways is of major relevance with respect to the possible mechanisms and consequences of thyroid-mediated immunomodulation in rainbow trout. Furthermore, the *atf1* mMPG was also found in the TSH signaling and in the thyroid hormone synthesis pathways (not shown), further pointing to a key role of this MPG in the thyroid-immune interaction.

3.4. Gene Set Enrichment Analysis (GSEA)

We performed GSEA as complementary approach to the mMPG methodology. Contrary to the focus on the genes with the largest change of expression, as done in the mMPG approach, GSEA uses the additional effects of small modulations of a set of genes (Subramanian et al., 2005) to identify those pathways with the most pronounced treatment-related modulations. The main difference between both methods lies in the fact that, while mMPG focus on the genes that are present in many pathways and evaluates the importance of each pathway by the number of its multipath genes that are modulated in response to T3/PTU, GSEA evaluates the overall modulation for every gene in each pathway.

Of originally 114 pathways or gene sets, only 31 fulfilled the required features to be analyzed by GSEA (minimum > 10 genes). Interestingly, the predominant gene sets that were most strongly enriched in both T3- and PTU-fed trout were the genes of NFkB and TLR pathways, suggesting that they were the main pathways modulated (See Fig. 4 for PTU-fed trout). Overall 11 gene sets were upregulated by PTU and only the complement and the coagulation gene cascades were downregulated (Table 3).

When comparing the pathways identified by the GSEA method with the mMPG method (Table 2), the NK, NFkB and TLR pathways were found to have 80, 52.2 and 41.6% of their genes modulated by PTU, respectively. This corroborates the results of the GSEA approach. In conclusion both methodological approaches, mMPG and GSEA, agree in the finding that the genes of NFkB, TLR and NK pathways appear to be among the most important targets of T3 or PTU in the trout immune system. It must be emphasized, however, that these pathways, although responsive to T3 or PTU, could also show alterations as a consequence of general physiological stress that could also being induced by the chemicals used in the treatments. Further studies will be necessary to going more in depth on the specificity and the extent of these responses.

3.5. Confirmation of microarray gene expression levels by RT-qPCR in selected genes

In order to validate the microarray results, mMPG were selected for RT-qPCR. In addition, two genes not present in the array but with key roles in several pathways (*creb, nfkb1*) were also mea-

sured by RT-qPCR (Table S1). The comparison between expression changes as found by the microarray method with those obtained by RT-qPCR (Fig. 5) shows that fold change responses were similar (correlation of $r = 0.892$, $p < 0.001$) for most genes (Fig. S1). The correlation indicates the reliability of the microarray data.

Creb expression in the PTU-exposed group was downregulated (0.645 fold change compared to that of control). On the other hand, T3 downregulated *nfkb1* (0.612 fold change). The CREB protein is a pleiotropic transcription factor, belonging to the same family as ATF1, that regulates a plethora of cellular processes including proliferation, differentiation and survival but moreover, it also appears to play a key role in immune responses (Rev. in Wen et al., 2010). In addition, CREB appears to have a mutual transcriptional antagonism with the thyroid hormone receptor (THR) (Mendez-Pertuz et al., 2003). One of the specific immune functions of CREB is to inhibit NF-kB activation through competition for limiting amounts of coactivators (CBP/p300). This means that the balance between CREB and coactivators would determine whether the overall response is inhibition (via CREB) or enhancement (via CBP/p300) of NF-kB signaling (Wen et al., 2010). Here, both treatments provoked a downregulation of *creb*, although it was significant only in the case of PTU. This is consistent with other studies which have shown that hypothyroidism resulting from iodine deficiency or PTU treatment significantly reduced total and phosphorylated CREB. However, so far the details of the mechanisms by which THs regulate CREB remain unsolved (Dong et al., 2009).

4. Conclusions

This study, to the best of our knowledge, is the first report describing immune gene transcript profiles induced by modulation of the thyroid status in rainbow trout in vivo. The use of a microarray enriched in immune-related genes present in the GenBank, in addition to those probes previously obtained from EST sequences, not only allowed the study of a larger number of rainbow trout immune-related genes than by using previous trout microarrays but also a higher reliability of the data because of their exact annotation. A first key observation from this study is that alterations of the in vivo thyroidal status of rainbow trout, as induced by treatment with either the active thyroid hormone T3, or the thyroid hormone synthesis inhibitor, PTU, resulted in altered transcript levels of a surprisingly high number of immune genes in the head kidney. This finding goes along with the pronounced expression of thyroid receptors in trout immune cells (Quesada-Garcia et al., 2014), and suggests that the thyroid system has a prominent immunomodulating role in trout. A second key observation is that PTU and T3 neither uniformly downregulated nor upregulated immune gene transcripts, but the effects varied with the individual genes. Thus, we should be cautious in assigning thyroid hormones to be immunostimulatory or immunosuppressive, but their role in the immune system appears to be rather complex and seems to vary with immune pathways and functions. Finally, by rational gene and pathway analysis, we could identify aftb1/jun and the NFkB and TLR pathways as crucial targets of thyroid activity in rainbow trout. With these results, there now exists a basis to investigate the functional implications of thyroid-mediated immunomodulation, and how environmental chemicals with thyroid-disrupting activity may interfere with proper immune function of fish.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aquatox.2016.02.016.

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