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**Perchlorate and Ethylenethiourea Induce Different Histological and Molecular Alterations in a Non-mammalian Vertebrate Model of Thyroid Goitrogenesis**

abbreviated title: “Thyroid Goitrogenesis in Amphibians”

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## Abstract

Despite evidence for a conserved role of thyroid-stimulating hormone (TSH) in regulating vertebrate thyroid function, molecular data on thyroid responses to TSH are mainly limited to mammalian species. In this study, we examined histological and molecular changes in the thyroid of *Xenopus laevis* tadpoles during a 12-day treatment with 20 mg/l perchlorate (PER) and 50 mg/l ethylenethiourea (ETU). Inhibition of thyroid hormone (TH) synthesis by PER and ETU was evident from developmental retardation, reduced expression of TH-regulated genes and up-regulation of *tshb-A* mRNA. Thyroid histopathology revealed goiters with strikingly different follicular morphologies following PER and ETU treatment. Using real-time PCR, we analyzed thyroids sampled on day 12 for differential expression of 60 candidate genes. Further temporal analyses were performed for a subset of 14 genes. Relative to the control, PER and ETU treatment modulated the expression of 51 and 49 transcripts, respectively. Particularly genes related to TH synthesis and protein metabolism were similarly affected by PER and ETU. However, several genes were differentially expressed in PER- and ETU-treated tadpoles. Specifically, goiter formation in the PER treatment was associated with low expression of genes related to DNA replication but high expression of negative growth regulators. Results from this work provide for the first time a characterization of gene expression profiles during goitrogenesis in a non-mammalian vertebrate model. Overall, our data suggest that, in addition to TSH over-stimulation, further mechanisms related to the mode of goitrogen action contribute to the regulation of thyroid gene expression.

## Introduction

Thyroid-stimulating hormone (TSH) is the primary physiological regulator of thyroid gland function and growth (Dumont et al., 1992a; Vassart and Dumont, 1992). In response to TSH stimulation, the thyroid tissue of all vertebrates produces and releases the same thyroid hormones (TH), thyroxine (T<sub>4</sub>) and 3,5,3'-triiodothyronine (T<sub>3</sub>), which play important roles for development, growth and metabolism (Leatherland, 1982; McNabb, 2007; Shi, 1999; Yen, 2001). Biosynthesis of TH is a multi-step process comprising iodide uptake by the sodium-iodide symporter (NIS), biosynthesis of thyroglobulin (TG), iodide organification by thyroid peroxidase (TPO) to yield iodothyronine-containing TG and finally, endocytosis and hydrolysis of iodinated TG to liberate TH (Dunn and Dunn, 2001). A hallmark of TSH action on thyroid follicular cells is to up-regulate the expression of many genes involved in TH synthesis and release (De Felice et al., 2004). Most effects of TSH on thyroid function are mediated by the intracellular second messenger cAMP, the generation of which is increased upon TSH activation of its membrane receptor (TSHR) (Vassart and Dumont, 1992).

In addition to the control of TH synthesis and release, TSH also stimulates thyroid growth *via* cAMP-dependent effects on thyroid cell proliferation (Kimura et al., 2001). The proliferative effect of TSH is reflected by the formation of hyperplastic goiters in response to chronic TSH over-stimulation (Dumont et al., 1992a). In line with the proposed role of cAMP in mediating the mitogenic effects of TSH, somatic mutations of TSHR or G<sub>s</sub>α proteins that constitutively activate the cAMP production by adenylate cyclase will result in a TSH-independent thyroid growth (Krohn et al., 2005). However, the signalling pathways involved in the mitogenic action of TSH are not yet fully understood, and there is evidence for species-specific differences in the control of thyroid growth by TSH and other growth factors (Bidey et al., 1999; Kimura et al., 2001).

From a comparative perspective, the central role of TSH for regulation of thyroid function and growth appears well conserved across vertebrate classes. Increases in radioiodide uptake and TH release, considered as paradigms of TSH-dependent functional responses, have been demonstrated in experimental animal studies with fish (Leatherland, 1982), amphibian (Shi, 1999), reptilian (Buckingham, 1970), and avian species (McNabb, 2007). Further, a conserved role of TSH for thyroid growth stimulation is suggested by numerous reports of thyroid hyperplasia following treatment of non-mammalian species with anti-thyroidal compounds (Bradford et al., 2005; Handa and Chiasson, 1980; Opitz et al., 2006a). However, although thyroidal responses to TSH have been well documented by biochemical and histological techniques, molecular analyses of thyroid tissue in non-mammalian species are still sparse (Elsalini et al., 2003; Grommen et al., 2006; Opitz et al., 2006b).

In anuran amphibians, TH play a pivotal role during metamorphic development by regulating diverse processes associated with organ and tissue transformation from larval to the adult phenotype (Furrow and Neff, 2006; Shi, 1999). We recently demonstrated the utility of metamorphic development in *X. laevis* tadpoles to serve as a novel non-mammalian model for investigations on TSH-induced alterations in thyroid gene expression (Opitz et al., 2006b). However, very few genes have so far been analysed for their expression profiles in the amphibian thyroid. For the amphibian model, we proposed that induction of thyroid goitrogenesis by means of anti-thyroidal treatment provides a promising approach to identify TSH-regulated genes under *in vivo* conditions. Based on the classic concept of thyroid system regulation, TH deficiency relieves the negative feedback of TH on pituitary TSH secretion resulting in elevated TSH plasma levels and a concurrent increase in TSH stimulation of the thyroid (Dumont et al., 1992b). The chronic over-stimulation of the thyroid gland by TSH

will finally result in goiter formation. Because iodide is essential for TH synthesis, diffuse hyperplastic goiters can be also induced by iodine deficiency (Denef, 1989). In fact, many mammalian models of thyroid goitrogenesis have used a combination of anti-thyroidal treatment and iodine deficiency (Denef, 1989). However, intracellular iodide levels are an important regulatory factor of thyroid follicular cell physiology modulating not only the effects of TSH on thyroid growth and function but affect thyroid function also in a TSH-independent manner (Eng et al., 1999; Maier et al., 2007; Scipioni et al., 2007). As a consequence, the thyroid phenotype obtained in experimental goiter studies reflects not only the over-stimulation by TSH but also thyroidal responses to altered states of iodide turnover. This hypothesis might help to explain the variable histological phenotypes of thyroid glands that were previously observed in *X. laevis* tadpoles treated with different anti-thyroidal substances (Opitz et al., 2006a; Opitz et al., 2006b; Tietge et al., 2005).

Given this background, we conducted a comparative analysis of the histological and molecular alterations in thyroids of *X. laevis* tadpoles treated with either PER to inhibit iodide uptake (Wolff, 1998) or ETU to inhibit iodide organification (Doerge and Takazawa, 1990). Results from these analyses confirmed previous observations of different histological phenotypes associated with each of the two treatment regimes (Opitz et al., 2006b). Moreover, for the first time, real-time PCR analyses provide information about expression profiles for a larger set of candidate genes in thyroid tissue of a non-mammalian vertebrate. Consistent with the observed histological phenotypes, the gene expression profiling revealed responses that were common to both treatments, particularly for genes related to TH synthesis/release and protein metabolism, but we also identified a subset of genes showing differential expression in response to PER and ETU. The molecular analysis of goitrous thyroids was supplemented by additional temporal analyses yielding further insights into the early events of goiter formation.

## Materials and Methods

### Animals and Husbandry

*Xenopus laevis* tadpoles were obtained from in-house breeding of adult animals. All experimental aspects were conducted in compliance with the institutional guidelines for the care and use of animals. Spawning of adult *X. laevis* was induced by injection of human chorionic gonadotropin (Sigma, Deisenhofen, Germany). Embryos and tadpoles were initially raised in a static renewal culture system ( $22 \pm 1^\circ\text{C}$ ; 12:12 h light:dark) as described in Opitz et al. (2005). After hatching, tadpoles were fed daily Sera Micron® (Sera GmbH, Heinsberg, Germany). Developmental stages were determined according to the criteria of Nieuwkoop and Faber (1994). At stage 46, tadpoles were transferred to a flow-through culture system (7.0 l glass tanks; flow rate of 25 ml/min) and were maintained in non-chlorinated tap water until they reached stage 51 on day 14 post-fertilization.

### Experimental Conditions

For experimental induction of goiter formation, three experimental treatment groups were established for the *in vivo* exposure experiment. Tadpoles were treated with either 20 mg/l PER (sodium perchlorate; Sigma) to block thyroidal iodide uptake or with 50 mg/l ETU (Sigma) to inhibit iodide organification, whereas tadpoles maintained in non-chlorinated tap water served as an untreated control group. A flow-through exposure system (tank volume of 7.0 l; flow-rate of 25 ml/min) was used to maintain continuous aqueous exposure of tadpoles

to the test chemicals during the experimental period of 12 days (Lutz et al., 2008). Samples (20 ml) of test solutions were collected from the treatment tanks on various dates and analyzed for actual aqueous concentrations of PER anion and ETU. Concentrations of PER in the test solution were determined by ion exchange chromatography using a 761 compact IC system (Metrohm, Filderstadt, Germany) equipped with a Metrosep A Supp 5 column (Metrohm). Concentrations of ETU were determined using a Gynotek 480 HPLC system equipped with a UV detector (Gynotek, Germering, Germany). ETU separation was performed on a Hyperclone ODS (C<sub>18</sub>) analytical column (Phenomenex, Aschaffenburg, Germany). On all time points analyzed, measured concentrations of PER and ETU did not differ by more than 10% from the nominal concentration of 20 and 50 mg/l, respectively (data not shown). The iodide concentration of the dilution water was 10 µg/l. Each of the three treatments consisted of four replicate tanks, and a total of 22 tadpoles were allocated to each of the 12 treatment tanks on study day 0. The experiment was conducted at 22 ± 1°C, pH 7.8 ± 0.2 and a 12:12 hours light:dark cycle. Tadpoles were fed Sera Micron® at a daily food ration increasing from 30 to 50 mg/animal during the course of the experiment.

On study days 1, 3, 5, 8, and 12, four to six animals were randomly selected from each tank and anesthetized in 100 mg/l aminoesterbenzoic acid (Sigma). Sampled tadpoles were assessed for developmental stage, whole body length (WBL) and hind limb length (HLL). Measurements of WBL and HLL were made using a SZX12 microscope (Olympus, Hamburg, Germany) equipped with a Color view III digital camera (Olympus). Morphometric analyses were performed using Analysis II software (Olympus). Pituitary and thyroid tissue was dissected from two to four animals per treatment tank, and tissue samples were separately collected in 15 µl of RNAlater reagent (Qiagen, Hilden, Germany). In addition, brain tissue was collected on study day 8 from two animals per tank for subsequent gene expression analysis. Tissue samples were stored at -80°C until RNA extraction. For thyroid gland histopathology, lower jaw tissue was sampled from two tadpoles per tank and fixed in Bouin's solution (Sigma) for 48 h.

### **Isolation of RNA and cDNA Synthesis**

Extraction of RNA from brain, pituitary and thyroid tissue was performed for tissue samples from individual animals, with the exception of thyroids sampled on day 1, where tissue samples from two animals were pooled before RNA extraction. Total RNA from pituitary and thyroid tissue was extracted using RNeasy Micro Kit (Qiagen) including the use of carrier RNA and on-column treatment with RNase-free DNase (Qiagen) according to the manufacturer's protocol. Total RNA from brain tissue was extracted using RNeasy Mini Kit (Qiagen) including on-column treatment with RNase-free DNase (Qiagen) according to the manufacturer's protocol. Total RNA content was measured using the RiboGreen RNA Quantitation Kit (Invitrogen, Karlsruhe, Germany) according to the manufacturer's protocol in a Spectrafluor Plus microplate reader (Tecan, Crailsheim, Germany). cDNA was synthesized from 50 ng total RNA in a 20 µl reaction using 5 pmol oligo(dT) primer (Biometra, Göttingen, Germany), 10 nmol of each dNTP (Biometra), and 1 µl of AffinityScript™ reverse transcriptase (Stratagene, Amsterdam, Netherlands). cDNA synthesis was performed according to the manufacturer's protocol (Stratagene) in a thermal cycler (Biometra). The cDNA was diluted 4- to 40-fold prior to PCR amplification.

### Real-time PCR

Relative quantifications of gene expression were performed using a MX3000 real-time PCR instrument (Stratagene). Transcript-specific primers (Supplemental Table 1) were designed using Clone Manager 7 software (Scientific & Educational Software, Cary, USA) and were synthesized by TibMolBiol (Berlin, Germany). Real-time PCR was carried out in 20  $\mu$ l reactions using 2  $\mu$ l of diluted cDNA solution as template. Target transcript-specific reactions contained 250-400 nM of each primer, 100 nM of each dNTP (Biometra), 1.5-3.0 mM MgCl<sub>2</sub>, 0.1  $\mu$ l of 200-fold diluted SYBR Green I solution (Invitrogen), ROX reference dye (Invitrogen), 1X reaction buffer, and one unit of Platinum Taq DNA Polymerase (Invitrogen). The thermal cycling protocols included an initial enzyme activation step at 95°C for 8 min followed by 40 cycles of denaturation (95°C, 17 s), annealing (62°C, 25 s) and extension (72°C, 25-30 s). The thermal amplification was followed by a dissociation-curve analysis to control for the specificity of the amplification products. The integrity of the PCR amplification was further validated by the detection of a single amplification product for each target transcript using gel electrophoresis and gel staining with ethidium bromide (GIBCO/BRL, Eggenstein, Germany). The specificity of all PCR reactions was confirmed by sequence analysis of PCR products using a CEQ 8800 Genetic Analysis System (Beckman Coulter, Krefeld, Germany).

For each set of transcript-specific primers, the efficiency of the PCR reaction was determined by analyzing five-fold serial dilutions (spanning at least three orders of magnitude) in triplicate. Efficiency of PCR ranged between 92 and 100% for the different target gene PCR assays. Relative values for target transcript abundance in individual samples were determined by using the comparative C<sub>T</sub> method ( $\Delta\Delta C_T$ ) according to Dorak (2006). Tissue-specific reference samples (calibrator) used to determine relative amounts of target transcript were created by pooling reverse transcribed RNA samples from different treatments. The relative quantities of target transcripts were determined with respect to the calibrator sample using corrections for PCR efficiency of the target gene assay and the normalizing gene assay. Relative abundance values of a target transcript were divided by the relative abundance value for the endogenous control transcript to achieve normalization for sample to sample variations in mRNA loading and RT-PCR efficiency. Because of its invariant expression in brain and pituitary tissue samples, rpl8 (ribosomal protein L8) mRNA was used for normalization of target gene expression in these two tissues. In thyroid tissue, however, expression of rpl8 tended to decrease in hyperplastic tissue samples. Therefore, eef1a (elongation factor 1 $\alpha$ ) mRNA was used for normalization of target gene expression in thyroid tissues. For each sampling time point, a total of eight RNA samples were analyzed per treatment group (biological replicates). For each target transcript, at least two independent PCR measurements (technical replicates) were performed per RNA sample and the data were averaged.

Several quality control measures were included in the PCR protocols to ensure the validity of the PCR experiments. Reverse transcription reactions performed without the RT enzyme served as negative controls to check for the absence of genomic contamination. PCR reactions performed with H<sub>2</sub>O replacing the cDNA template served as additional negative controls to check for the specificity of target cDNA amplification. Furthermore, in order to account for the increased variability detected between replicate measurements at high C<sub>T</sub> values (> 35 cycles), we restricted quantification of expression values to those samples where the C<sub>T</sub> value for a given target gene was <35. Samples where the C<sub>T</sub> value for a given target gene ranged between 35 and 40 were designated as having detectable expression 'below the limits of

quantification'. Samples where the  $C_T$  value for a given target gene was  $> 40$  were designated as having no detectable expression.

### **Thyroid Gland Histology**

Lower jaw tissue samples containing the paired thyroid gland fixed in Bouin's fixative were dehydrated in a graded series of alcohol, embedded in Histoplast S (Serva, Heidelberg) and sectioned in a horizontal plane from ventral to dorsal at 3 to 4  $\mu\text{m}$  thickness. Serial sections were mounted on glass slides and stained with periodic acid-Schiff (PAS) and Mayer's hematoxylin.

### **Data Analysis**

Developmental stage data were analyzed by Dunn's multiple comparison test to determine significant differences in stage distribution between treatment groups. All other data sets were first analyzed for normal distribution (Kolmogorov-Smirnov test) and homogeneity of variances (Levene test). If measurements of a response attribute, or a log-transformation of it, were found to be normally distributed with equal variances, then Tukey-Kramer's multiple comparison test was used for pair-wise comparisons between treatments. In cases where transformation was not successful to achieve normality and equal variances, Dunn's multiple comparison test was applied. Statistical analyses were performed using the software package GraphPad Prism 4.0 (GraphPad, San Diego, CA, USA). Differences were considered significant at  $p < 0.05$ .

## **Results**

### **Organismal Responses to PER and ETU Treatment**

Organismal responses to PER and ETU treatment were assessed by stage determination as well as morphometric measurements of WBL and HLL of animals sampled on study days 1, 3, 5, 8, and 12 (Fig. 1). An initial analysis showed that there were no tank effects within treatments for any of the three organismal endpoints and therefore, data from replicate tanks were pooled within treatments for final statistical analyses. No significant differences between treatments were detected for WBL on any sampling day. No significant differences in developmental endpoints (developmental stage, HLL) were observed between treatments on study days 1, 3, 5, and 8. On study day 12, however, all control tadpoles showed development to stage 56 whereas PER and ETU treatment caused developmental arrest of tadpoles at stages 54/55. Morphometric analyses of HLL confirmed the inhibitory effect of PER and ETU on metamorphic development since mean HLL values of tadpoles treated with PER and ETU were significantly lower than controls on day 12. Together, the apical morphological measurements confirm a specific inhibition of TH-dependent development but not of tadpole growth by goitrogen treatment.

### **Treatment with PER and ETU Alters Gene Expression in Tadpole Brain**

Using real-time PCR, the expression of TH-regulated genes was analyzed in tadpole brain in order to assess the presence of hypothyroid conditions at the molecular level. Brain tissue from stage 55 tadpoles sampled on study day 8 was used for these analyses to prevent possible confounding effects of stage-dependent differences in gene expression. In brain of PER- and ETU-treated tadpoles, significantly lower mRNA expression was detected for

several genes known to be up-regulated by TH (Das et al., 2006; Denver et al., 1997) including *thrb* (thyroid hormone receptor  $\beta$ ), *bteb1-A* (basic transcription element-binding protein 1), *pcna*, *mcm2*, and *kif2C* (Fig. 2). For *dapl1*, a gene down-regulated by TH in tadpole brain (Das et al., 2006), mRNA expression was increased in brain of goitrogen-treated tadpoles (Fig. 2). Notably, brain gene expression was altered by PER and ETU treatment in a similar manner. Combined with the morphological observations, these data validate the experimental design by confirming that both treatments were similarly effective in causing hypothyroidism.

### **Expression of *tshb-A* mRNA is Increased in Tadpoles treated with PER and ETU**

Temporal profiles of *tshb-A* mRNA expression were analyzed by real-time PCR using total RNA extracted from micro-dissected pituitaries of tadpoles sampled in the different treatment groups (Fig. 3). On study day 1, *tshb-A* levels were similar in all three treatment groups. Between study day 1 and 12, *tshb-A* expression increased 2.1-, 3.7- and 4.1-fold in the control, PER and ETU groups, respectively. When expression levels were compared between tadpoles matched for sampling time, increased *tshb-A* expression relative to the control group was detected in PER-treated tadpoles on study days 5, 8, and 12 and in ETU-treated tadpoles on study days 8 and 12 (Fig. 3). Thus, a lag period of 5 to 8 days preceded the detection of significant increases in *tshb-A* expression in goitrogen-treated tadpoles.

### **Treatment with PER and ETU Cause Differential Effects on Thyroid Histology**

Differences in the histological appearance of thyroid glands after longer-term treatment with PER and ETU have previously been described for *X. laevis* tadpoles (Opitz et al., 2006b; Tietge et al., 2005). The present study therefore aimed at comparing the initial histological changes during thyroid goitrogenesis induced by PER and ETU (Fig. 4). On study day 1, thyroids of control tadpoles were composed of small follicles with flattened epithelial cells enclosing a PAS-positive colloid. A progressive increase in glandular size was observed in control tadpoles during the experimental period of 12 days. The glandular diameter increased from approximately 100 -150  $\mu\text{m}$  in stage 51/52 tadpoles (study day 1) to approximately 250 - 400  $\mu\text{m}$  in stage 56 tadpoles (study day 12). This increase in overall glandular size was accompanied by increases in follicle number as well as increases in size of individual follicles. Follicular cell height increased markedly from day 5 onwards. On study day 12 (Fig. 4E), thyroid follicles of the controls were composed of cuboidal and low columnar epithelial cells enclosing a PAS-positive colloid with minimal peripheral vacuolation.

Thyroid size of tadpoles treated with PER and ETU was similar to controls on study days 1 and 3 (Fig. 4). A moderate thyroid enlargement was observed on study day 5 in both goitrogen groups and became more prominent after 8 days of treatment. By study day 12, PER- and ETU-treated tadpoles presented thyroids that we characterized as small goiters because of a several-fold increase in size relative to time-matched controls (Fig. 4J,4O). During the time course of the experiment, a distinct sequence of histological changes was observed in thyroids of goitrogen-treated tadpoles. Early histological changes comprised a mild to moderately severe depletion of stainable colloid within 3 to 5 days of treatment, respectively, followed by follicular cell hypertrophy within 5 days and follicular cell hyperplasia within 8 days of goitrogen treatment.

Despite the similar temporal sequence of specific histological alterations, the follicular architecture of thyroids was markedly different between the PER and ETU treatment (Fig. 4). In thyroids from PER-treated tadpoles (Fig. 4J), colloid depletion was associated with a

marked reduction in the luminal space, and collapsed follicles containing very little stainable colloid were a characteristic feature in these thyroids. In contrast, ETU treatment resulted in a progressive enlargement of follicles with distended luminal cavities containing a faintly staining material upon PAS staining (Fig. 4O).

### Differential Expression of Candidate Genes in Thyroid Tissue

A candidate gene approach was used to characterize profiles of thyroid gene expression in tadpoles from PER, ETU, and control treatments. Based on a review of published literature, candidate genes were selected according to their putative role or functional relevance for specific cellular processes related to TH synthesis, secretion and metabolism, protein biosynthesis and vesicular transport, cell growth and proliferation, angiogenesis, and stress responses. As a first step, we determined the relative expression of 60 candidate genes in thyroid tissues sampled on study day 12 (Table 1). In a second step, a subset of candidate genes was further characterized for temporal expression profiles in the different treatment groups between study day 1 and 12.

### Expression Profile on Day 12

*Overview.* Multiple comparisons among the three experimental groups revealed that a high proportion of the genes analyzed (56 out of 60 genes) was differentially expressed between at least two experimental groups (Table 1). Only four genes (c-myc I, c-myc II, vegf-A, kdr-a) did not show significant differences in their expression between treatment groups. Compared to the control group, a total of 51 transcripts showed a differential expression in PER-induced goiters, including 41 genes with significantly increased expression and 10 genes with significantly decreased expression in the PER group. In ETU-treated tadpoles, thyroidal expression of 49 genes was significantly different from the control group, including 43 genes with increased expression and 6 genes with decreased expression in the ETU group. We also identified 13 genes with significant differences in their mRNA expression between the two goitrogenic treatments.

*TH Synthesis, Secretion and Metabolism.* Several genes related to TH synthesis (slc5a5, tpo, tg, tshr), TH secretion (ctsb), and TH metabolism (dio1, dio2, dehal1) showed increased expression in goitrous thyroids from the PER and ETU treatment groups. Notably, the strongest up-regulation found in the present survey was detected for slc5a5 mRNA in the PER treatment (62.5-fold increase over control level). Up-regulation of slc5a5 mRNA by ETU was also very strong (32.2-fold) but significantly lower than in the PER treatment. Heterogeneous alterations were detected with regard to the regulation of iodothyronine deiodinases dio1, dio2, and dio3 in goitrous thyroids. While expression of dio2 mRNA was up-regulated by both goitrogen treatments, dio1 expression was only up-regulated by PER. In contrast, expression of dio3 mRNA declined to very low levels in thyroids of PER- and ETU-treated tadpoles.

*Protein Biosynthesis and Vesicular Transport.* In goitrous thyroids, a large number of genes related to protein biosynthesis, processing and transport were up-regulated. A general finding for this group of genes was that the detected changes in expression occurred in a qualitatively and quantitatively similar fashion in PER- and ETU-treated tadpoles. The list of candidate genes related to RNA translation comprised initiation factors (eif4a1, eif4e), termination factors (etf1, gspt1, gspt2) and a gene involved in ribosome biogenesis (nolc1). In addition, all candidate genes related to endoplasmic reticulum (ER) function (hsps5, pdia6, xbp1, ero11, edem1) and protein folding in mitochondria (hspd1) were up-regulated by PER

and ETU treatment. Further, an increased abundance of transcripts involved in vesicular protein transport was detected in goitrous thyroids including mRNAs encoding for proteins with well-conserved functions during COPII vesicle formation (*sar1a*, *rnp24*, *sec24c* and *copa*) and endocytosis (*rab5a*, *rab7*).

*Cell Cycle.* Genes related to cell proliferation showed only weak to moderate changes in expression (less than 2-fold up- or down-regulation) following 12 days of goitrogen treatment. Despite the small changes detected, low sample-to-sample variation still allowed for the detection of significant differences in the expression level of various candidate genes. For two transcripts (*c-myc I*, *c-myc II*), relative expression levels were not altered. Thyroids from PER-treated tadpoles had significantly lower expression levels of DNA replication marker genes (*pcna*, *mcm2*, *mcm5*, *ccnd1*, *aurkb*) compared to ETU-treated tadpoles and for two transcripts (*mcm5*, *ccnd1*), expression levels were even reduced if compared to control animals. In contrast, five genes were significantly up-regulated by ETU treatment (*pcna*, *mcm2*, *aurkb*, *kif2C*, *chc1-a*) relative to controls. Expression of *igf2* mRNA was significantly up-regulated following PER and ETU treatment, whereas expression of *igf1* decreased to very low levels in PER-treated tadpoles ( $C_T$  values  $> 35$ ; detectable but below the limit of quantification) and was not detectable ( $C_T > 40$ ) in 6 out of 8 thyroid samples from the ETU treatment group.

*Growth Arrest and Apoptosis.* Given the dissimilar expression of several cell cycle-related genes following PER and ETU treatment, it was interesting that differential responses to PER and ETU treatment were also detected for a subset of genes related to negative growth regulation (*gadd153*, *gadd45g*, *sesn1*). Up-regulation of these transcripts was either specific to PER treatment (*gadd153*, *sesn1*) or more marked in the PER treatment (*gadd45g*). However, expression of still other genes related to growth arrest (*gadd45a*, *gas6*, *dapl1*) and apoptosis (*bcl-XL*, *bag1*) was similarly affected by PER and ETU treatment.

*Cellular Stress Response.* Goitrous thyroids were further characterized by increased expression of several transcripts implicated in cellular stress responses (*nrf2*, *sod2*, *cat*, *gstm2*, *hif1a*). Up-regulation of these genes occurred in both goitrogen treatments in a similar fashion. In contrast, a differential regulation of *gstp1* mRNA expression was observed in tadpoles treated with PER (down-regulation) and ETU (up-regulation).

*Angiogenesis.* Although gross examination of thyroids from goitrogen-tadpoles revealed a strong vascularization, only few significant changes in expression were detected for genes implicated in angiogenesis. Expression of *vegfa* and *kdr-a* was not significantly affected at the mRNA level by goitrogen treatment whereas reduced expression was detected for the pro-angiogenic factor *ang1* in the PER treatment and the endothelial cell marker *tie-2* in both goitrogen treatments.

*Miscellaneous.* Treatment with PER and ETU also affected other genes related to polyamine metabolism (up-regulation of *odc-1*), amino acid synthesis (up-regulation of *asns* by PER only), and tight junction function (down-regulation of *cldn6*).

### **Temporal Gene Expression Profiles during Goiter Formation**

*Control Profile.* For a subset of 14 genes, we also analyzed the temporal expression profile in the different treatment groups based on thyroid tissue samples taken on study days 1, 3, 5, 8 and 12 (Fig. 5-8). A first statistical analysis addressed possible temporal changes in control gene expression levels during the experimental period of 12 days and detected significant changes in expression of three genes (*slc5a5*, *hspa5*, *gstp1*). Significant changes in the control group included a 2.5-fold increase of *slc5a5* mRNA between day 1 and day 12, a 2.9-fold

increase of hspa5 mRNA on day 8 and day 12 relative to day 1 as well as a significantly increased expression of gstp1 mRNA (10.5- to 12.6-fold) on days 3, 5, 8, and 12 relative to day 1.

*TH Synthesis.* The three genes related to TH synthesis (slc5a5, tpo, tshr) showed a very similar temporal expression profile which was characterized by a lag period of three and five days until a significant up-regulation was evident in tadpoles treated with PER and ETU, respectively (Fig. 5). For these transcripts, expression levels progressively increased in goitrogen-treated tadpoles until study day 12. Notably, mRNA expression of slc5a5 but not tpo or tshr was significantly higher in tadpoles treated with PER compared to ETU on study days 5, 8, and 12.

*Protein Metabolism and Vesicular Transport.* Different temporal expression profiles were detected when analyzing four candidate genes (eif4a1, hspa5, sar1a, rnp24) related to protein metabolism and transport (Fig. 6). Expression of eif4a1 and hspa5 mRNAs was significantly up-regulated within 24 h of goitrogen treatment and their expression remained elevated throughout the experimental period of 12 days. The expression profile of sar1a closely resembled the pattern found for slc5a5, tpo, and tshr as up-regulation of sar1a mRNA was only observed after a lag period of 3 and 5 days in PER- and ETU-treated tadpoles, respectively. In comparison, rnp24 represented a late response gene showing significant up-regulation only after 8 and 12 days of goitrogen treatment.

*Cell Proliferation.* The temporal profiles determined for the DNA replication markers pcna and mcm2 differed between the two goitrogen treatments (Fig. 7). Both transcripts were rapidly up-regulated in thyroids by PER and ETU treatment, mcm2 mRNA within 24 h and pcna mRNA within 3 days. However, at later time points, elevated expression was maintained only in ETU-treated tadpoles. In tadpoles treated with PER, the early upregulation of mcm2 and pcna expression was transient and reduced expression levels were detected on day 8 (significantly reduced compared to control and ETU groups) and day 12 (significantly reduced compared to ETU group).

*Angiogenesis.* We also analyzed the temporal expression of two pro-angiogenic genes (vegf-A, tie-2) during goiter formation (Fig. 7). Mean values for vegf-A mRNA expression showed some conspicuous increases early during goitrogen treatment, but the differences were not statistically significant, partly due to high sample-to-sample variation of expression levels. In contrast, relative expression levels of tie-2 mRNA were reduced after a lag period of 8 and 5 days in the PER and ETU treatment groups, respectively.

*Compound-Specific.* Two genes related to growth arrest (gadd153) and amino acid synthesis (asns) shared a very unique temporal expression profile (Fig. 8). Marked up-regulation of both genes was limited to PER treatment and occurred temporally in a very similar fashion with significant changes being observed from day 5 onwards. Another gene with a very compound-specific expression pattern was gstp1 (Fig. 8). Up-regulation of gstp1 mRNA was restricted to ETU treatment and was detectable from day 5 onwards. In contrast, a rapid down-regulation of gstp1 mRNA levels was detected in PER-treated tadpoles. Compared to the control group, gstp1 expression declined to 5.5- and 9-fold lower expression levels within 24 h and 3 days of PER treatment, respectively. Expression of gstp1 mRNA in PER-treated tadpoles increased transiently to control levels on days 5 and 8 but was again significantly reduced on day 12.

## Discussion

In the present study, we characterize for the first time the gene expression changes during experimental induction of thyroid goiters in a non-mammalian vertebrate. The specific experimental design of our studies allowed us to address several important aspects of thyroid goitrogenesis including the evaluation of gene response profiles in a comparative, developmental, temporal and goitrogen-specific context.

In our experimental model, treatment of *X. laevis* tadpoles with goitrogenic substances is initiated at premetamorphic stage 51, that is shortly after functional maturation of the thyroid gland (Regard, 1978). The rationale for goitrogen treatment was to induce hypothyroid conditions leading to increased TSH synthesis/release by the pituitary and a chronic over-stimulation of the thyroid gland by TSH. It is well established that feedback along the hypothalamus-pituitary-thyroid axis is operative in amphibian tadpoles undergoing metamorphosis (Manzon and Denver, 2004). Given that analyses of circulating plasma levels of T<sub>3</sub>, T<sub>4</sub>, and TSH are not feasible for this small animal model, we used a panel of alternative endpoints to demonstrate the presence of hypothyroid condition in goitrogen-treated tadpoles. Metamorphic development is a TH-dependent process and assessment of morphological development in the differentially treated tadpoles provided a simple means in our model to confirm the inhibitory activity of PER and ETU on development. The tadpole brain is a key target of TH action during the developmental period covered by the chemical treatment (Das et al., 2006; Helbing et al., 2007), and our analysis of gene expression in tadpole brain tissue corroborated the presence of TH deficiency. Further evidence was obtained from the detection of increased *tshb-A* expression in the pituitary of tadpoles treated with PER and ETU. Collectively, the selected morphological and molecular endpoint responses confirmed the expectation that both treatments were effective in inducing TH-deficient conditions in the test animals.

While goiter formation in amphibian larvae has previously been investigated after longer-term treatment with PER and ETU (Miranda et al., 1996; Opitz et al., 2006a; Tietge et al., 2005), a characterization of early changes in thyroid histology was performed in the present study. The temporal sequence of specific histological alterations was similar for thyroid glands of tadpoles treated with PER and ETU and included increased colloid resorption, follicular cell hypertrophy, thyroid hyperplasia, and goiter formation within 3, 5, 8, and 12 days of treatment, respectively. These findings in *X. laevis* correlate well with other reports on experimental induction of hyperplastic goiters in mammalian species (Denef et al., 1981; Wynford-Thomas et al., 1982b). However, thyroid histopathology also revealed that PER and ETU treatment cause goiters with strikingly different follicular morphologies. In PER-treated tadpoles, colloid depletion was accompanied by a concurrent reduction in the size of follicular lumina. The majority of the hyperplastic follicles in the PER treatment were characterized by narrow, slit-like or collapsed lumina. A similar histological appearance has been reported for thyroids of other species treated with PER (Stoker et al., 2006) or iodine-deficient diets (Denef et al., 1981). In sharp contrast, colloid depletion was accompanied by distension of follicular lumina in ETU-treated tadpoles. Furthermore, ETU-induced goiters in tadpoles had a more heterogeneous histological appearance, similar to what has been reported in dogs after treatment with ETU (Kameda et al., 1984). The mechanisms involved in the induction of different follicular morphologies are still unknown, but these observations support the view that in addition to the level of TSH stimulation, goiter development is modulated by still other factors, one of which might include mechanism of action-related differences in thyroidal iodide turnover (Denef, 1989; Dumont et al., 1992b).

The major aim of the current study was to provide a first comparative analysis of gene expression patterns during chemical-induced goitrogenesis in a non-mammalian vertebrate. Given the almost complete absence of gene expression data for the amphibian thyroid, we decided to exploit the high precision of real-time PCR assays for thorough analyses of temporal changes in favor of a single time point microarray study. For the present study, we further focused our analyses on a list of 60 candidate genes, selected for their important role in cellular processes that are expected to be relevant for thyroid function, growth and development.

Results from previous biochemical and histological studies in different vertebrate species suggest a conserved role of TSH for the regulation of thyroid function (Dumont et al., 1992a, McNabb, 2007; Regard, 1978). The present study demonstrates clearly that a conservation of major thyroid cell responses to TSH stimulation is also apparent at the level of thyroid gene expression. Goitrogen treatment resulted in altered expression of many genes involved in TH synthesis and iodine metabolism. Notably, well-established TSH-responsive genes in mammalian systems such as *slc5a5*, *tpo*, *tg*, *dio2*, *dehal1*, and *ctsb* (Calebiro et al., 2006; De Felice et al., 2004; van Staveren et al., 2006a; Yamada et al., 2006) consistently showed increased expression in thyroids of goitrogen-treated tadpoles. The most marked increase was detected for *slc5a5* mRNA (encoding for NIS) being increased in PER-treated tadpoles more than 60-fold over control level. Moreover, *slc5a5* mRNA expression in the PER treatment was also significantly higher compared to ETU. Given that overall gene expression changes did not indicate goitrogen-specific differences in TSH stimulation, a possible explanation could be that the differential *slc5a5* mRNA expression reflects differences in intrathyroidal iodide turnover between PER and ETU treatments. Auto-regulation of NIS expression by iodide has previously been demonstrated (Eng et al., 1999; Glatt et al., 2005; Uyttersprot et al., 1997) and included down-regulation of NIS expression by increased iodide levels. On the other hand, NIS expression has been found to be increased by iodine deficiency, an effect that appears to occur in a TSH-independent manner (Maier et al., 2007; Scipioni et al., 2007). Thus, auto-regulatory mechanisms are likely to contribute to the regulation of NIS expression in the amphibian thyroid.

Results from the expression survey revealed also differences between established mammalian systems and the amphibian model. Of particular interest is the persistent up-regulation of *tshr* mRNA in thyroids of goitrogen-treated tadpoles. Such a positive regulation of *tshr* expression by TSH is different to what has been found in most mammalian model systems, where chronic TSH over-stimulation was associated with decreased TSHR expression (Glatt et al., 2005; Lalli and Sassone-Corsi, 1995), the latter being regarded as one of several intracellular feedback mechanisms attenuating cellular responses to chronic TSH stimulation (van Staveren et al., 2006b; Wynford-Thomas et al., 1982a). This intracellular negative feedback mechanism might not be active in the tadpole thyroid, at least not at the level of *tshr* mRNA expression. In turn, a positive TSH regulation of *tshr* mRNA expression could provide a physiologically relevant mechanism to maintain and enhance the responsiveness of the tadpole thyroid to the progressively increasing TSH levels at late stages of metamorphic development. Particularly at climax stages, rapid increases in the rates of TH synthesis/release are required to provide the high TH levels necessary for initiation of a late program of tissue and organ remodelling (Shi, 1999). The temporal analysis of functional marker gene expression (*slc5a5*, *tpo*, *tshr*) revealed a lag period of three to five days until significant up-regulation was detectable in tadpoles treated with PER and ETU, respectively. The faster induction by PER might be due to pharmacokinetic differences between PER and

ETU, but the contribution of additional mechanisms related to the mode of action cannot be ruled out. For the amphibian model, these temporal data suggest that it requires more than 24 h of goitrogen treatment to significantly alter the level of thyroidal TSH stimulation.

Our analyses also provided new insights into the regulation of iodothyronine deiodinase expression in the vertebrate thyroid. When combined with data from other species, our data corroborate the view that thyroidal expression profiles as well as hormonal regulation of deiodinase expression are highly species-specific. First, we were able to detect mRNA expression for all three deiodinases in the amphibian thyroid. Further, experiments using different primer pairs suggest that mRNA expression of *dio2* is higher than for *dio1* and *dio3*, at least at the developmental stages examined in this study (data not shown). High expression of DIO2 mRNA has been detected in human, but not rat thyroid glands, and only human DIO2 expression is increased by TSH (Gereben et al., 2001; Köhrle et al., 2005; Yamada et al., 2006). Thus, our observation of increased *dio2* expression in the amphibian thyroid after treatment with PER and ETU suggests similar regulation as reported for the human thyroid (Köhrle et al., 2005; Yamada et al., 2006). In the rat thyroid, *Dio1* mRNA is abundantly expressed and its expression is increased by TSH in a cAMP-dependent manner (Köhrle et al., 2005). In tadpoles, however, *dio1* expression was increased by PER but not by ETU suggesting that TSH might not be the primary regulator in *X. laevis* thyroids. One clue to the understanding of the PER-specific effect on *dio1* expression could include consideration of auto-regulatory effects of iodide (Pedraza et al., 2006). Very little is known about *dio3* expression in the vertebrate thyroid. Low mRNA expression has been detected in human thyroid (Yamada et al., 2006) as well as in thyroid tissue of chicken embryos (van der Geyten et al., 2002) but not in the rodent thyroid (Köhrle et al., 2005). In the amphibian thyroid, *dio3* mRNA was clearly detectable in control tadpoles but was strongly down-regulated by PER and ETU treatment. At present, the molecular mechanisms involved in regulation of thyroidal *dio3* expression are not known. Given that *dio3* mRNA is up-regulated by TH in various tadpole tissues during amphibian metamorphosis (Furlow and Neff, 2006), it will be important to consider both TH and TSH as putative hormonal regulators of thyroidal *dio3* expression. Studies using *ex vivo* organ culture of thyroid glands are currently in progress to yield further insights into the hormonal regulation of iodothyronine deiodinase expression in the amphibian thyroid. A peculiar characteristic of our amphibian model is that the period of goitrogen-induced TSH over-stimulation covers a late phase of thyroid development. In analogy to the differential effects caused by TH in developing tadpole tissues and in terminally differentiated tissues of adult frogs (Shi, 1999), it will also be of paramount interest to study responses of thyroid tissue in adult animals before generalized conclusions can be drawn.

The increased expression of genes related to RNA translation, post-translational protein modification and vesicular protein transport in goitrous thyroids is concordant with the induction of hypertrophy/hyperplasia during goitrogen treatment as promotion of protein synthesis/metabolism is a well-established requirement for cell growth and proliferation. The up-regulation of RNA translation factors indicates that one arm of the TSH-induced cellular response in the amphibian thyroid aims at augmenting global translation rates. The up-regulation of various genes implicated in ER function and vesicular protein transport is likely related to another major hallmark of TSH action, namely the stimulation of synthesis and secretion of TG, the main secretory product of thyroid follicular cells (Dunn and Dunn, 2001). The expression signature detected for transcripts related to ER function (*hspa5*, *pdia6*, *ero11*, *xbp1*, *edem1*) is consistent with the critical role played by the ER for TG folding and

maturation (Arvan et al., 1997; Baryshev et al., 2004; Sargsyan et al., 2004). In both amphibian and mammalian models of thyroid goitrogenesis, commonly described ultrastructural phenotypes include a dramatic expansion of rough ER in thyroid follicular cells (Miranda et al., 1996; Olen, 1969). Notably, we detected a well-conserved pattern of gene expression related to activation of ER stress response pathways, a cellular programme adjusting ER capacities for protein folding to different physiological conditions (Ron and Walter, 2007). Altered protein metabolism was also evident from the increased expression of genes encoding for components of COPII-coated vesicles (*copa*, *sar1a*, *sec24c*, *rnp24*) mediating the ER-to-Golgi transport of proteins. Very strong up-regulation was evident for *sar1a* mRNA encoding for a small G-protein of the Ras superfamily that plays a critical role in COPII-coated vesicle formation (Lee and Miller, 2007). To date, very little was known about thyroidal *Sar1a* expression (Baris et al., 2004), but the marked *sar1a* induction observed in goitrous thyroids might provide one clue to understand how the TSH-regulated increase of TG export rates from the ER is mediated at the molecular level (Arvan et al., 1997). In fact, the expression patterns detected for *sar1a* mRNA during goiter formation in this study, when coupled to the reported cAMP-responsiveness of the *Sar1a* gene (Zambon et al., 2005), suggests that *sar1a* might represent a novel TSH-response gene.

Our initial analysis of day 12 samples for expression of transcripts involved in DNA replication and cell cycle regulation revealed only mild differences between hyperplastic goiter samples and control thyroids, possibly due to a relatively high basal proliferative activity in the control thyroids masking the stimulatory effects of goitrogens on proliferation marker genes. On the other hand, at least for the DNA replication marker genes, *mcm2* and *pcna*, timing of tissue sampling appeared critical for the assessment of goitrogenic effects, because elevated expression was clearly detectable at early time points during goitrogen treatment, well before hypertrophy and hyperplasia were observed at the histological level. Interestingly, PER- and ETU-induced goiters showed different expression signatures of genes involved in cell proliferation. A lower expression of DNA replication marker genes (*pcna*, *mcm2*, *mcm5*, *ccnd1*, *aurkb*) as detected in PER-treated tadpoles on day 12 was concordant with a smaller size of PER-induced goiters compared to ETU. Further, *mcm2* and *pcna* RNA expression in PER-treated tadpoles was only transiently up-regulated at early stages of goitrogenesis. This is in line with the observation that ETU-induced goiters continue to increase in size for prolonged periods of time (Opitz et al., 2006a), whereas in PER-treated tadpoles, goiter growth is readily slowed after an initial phase of glandular enlargement and these goiters also never attain the very large size of those resulting from ETU (Opitz et al., 2006a) or propylthiouracil treatment (Coleman et al., 1967). Although previous studies with rodents reported about differential goiter growth following treatment with PER and thionamides (Ortiz-Caro et al., 1983), our study is the first to address possible molecular mechanisms associated with these differential responses to goitrogen treatment.

Insulin-like growth factors (IGFs) and vascular endothelial growth factors (VEGFs) are two physiologically relevant groups of growth factors that co-operate with TSH to regulate thyroid cell proliferation and microvascular remodelling (Bidey et al., 1999; Viglietto et al., 1997). Expression profiles detected in the amphibian thyroid suggest species-specific differences of thyroidal growth factor expression following goitrogen treatment. In the tadpole thyroid, *igf1* mRNA expression was very low and in contrast to human, dog and rat models (Kimura et al., 2001), goitrogen treatment of tadpoles did not stimulate but further reduced thyroidal *igf1* expression. In contrast, thyroidal *igf2* mRNA expression was increased in a similar fashion by PER and ETU treatment of tadpoles. Although vascular changes were

prominent in thyroids from goitrogen-treated tadpoles, both at the gross morphological and histological level, mRNA expression levels of vegf-A and its tyrosine kinase receptor kdr-a were not significantly altered. This lack of altered expression is different to many mammalian studies reporting up-regulation of thyroidal Vegf mRNA levels during goiter formation and TSH stimulation of thyroidal VEGF synthesis *in vitro* (Sato et al., 1995; Viglietto et al., 1997). It should be noted that this still limited analysis of growth factor expression did not explain the differential thyroid growth.

Concerning the differential goiter morphologies, a key finding of our expression profiling was that PER treatment resulted in increased expression of several transcripts encoding for negative growth regulators such as gadd153, gadd45a, gadd45g and sestrin. One of the induced transcripts encodes for the transcription factor gadd153, also known as CHOP (CCAAT/enhancer-binding protein-homologous protein), which has been shown to induce growth arrest and apoptosis in response to various cellular stress conditions including ER stress, oxidative stress, hypoxia, amino acid depletion and DNA damage (Oyadomari and Mori, 2004). In tadpole thyroids, up-regulation of gadd153 mRNA was not only specific to PER treatment but also coincided temporally with diminished expression of DNA replication markers. Thus, gadd153 up-regulation might be involved in mediating the reduced growth response of PER-treated thyroids. The molecular mechanisms regulating Gadd153 expression in the thyroid are not fully clear. Although Gadd153 mRNA and protein are induced by TSH in FRTL-5 cells (Pomerance et al., 2003; Sargsyan et al., 2004), Gadd153 appears not to be direct TSH response gene and its induction is likely a secondary response to TSH-induced cellular stress conditions. Our *in vivo* data are in line with this view because ETU treatment did not alter gadd153 expression and in PER-treated tadpoles, gadd153 induction was delayed relative to slc5a5 and tpo. In the tadpole thyroid, the expression pattern of asns mRNA closely matched that of gadd153. Common to both the gadd153 and asns promoters is the presence of a C/EBP-ATF composite site which is known to be activated in response to various stress conditions via a well characterized pathway including phosphorylation of eukaryotic initiation factor-2 $\alpha$  (Bruhat et al., 2002). The nature of the putative stress signal triggering this pathway in thyroids of PER-treated tadpoles is still unknown.

Further evidence for the existence of goitrogen-specific cellular stress responses comes from the observation of markedly different gstp1 mRNA expression profiles following treatment with PER (down-regulation) and ETU (up-regulation). Glutathione transferases (GSTs) play important roles in antioxidant cell defense and maintenance of cellular redox state. Recent studies showed elevated Gstp1 mRNA expression in thyroids of Wistar rats following propylthiouracil treatment (Glatt et al., 2005) and increased GST-pi expression along with other antioxidant enzymes in human benign thyroid nodules (Krause et al., 2007; Srisomsap et al., 2002). Given that Gstp1 expression is redox-sensitive and induced by oxidative stress in various cell types (Xia et al., 1996), up-regulation of gstp1 mRNA, as seen in ETU-treated tadpoles, might provide a novel marker for oxidative stress in the amphibian thyroid. In contrast, the very rapid down-regulation of gstp1 mRNA during PER treatment is more difficult to explain as an extensive literature search revealed no study reporting on a similar down-regulation of GST-pi in thyroid or other cell types. The unique de-regulation of gstp1 by PER might be even more appreciated in the light of recent studies showing that GST-pi, independent from its enzyme activities, plays an important role in the coordinated regulation of various stress kinases (e.g., c-Jun NH<sub>2</sub>-terminal kinase) and stress kinase-associated proliferation pathways (Adler et al., 1999; Ruscoe et al., 2001).

In combination, our histological and molecular analyses in *X. laevis* tadpoles link for the first time the induction of different thyroid morphologies caused by the NIS inhibitor PER and the TPO inhibitor ETU with the detection of compound-specific differences in thyroidal gene expression. The present data are important because they clearly show that different goitrogens vary in their potential to alter the expression of specific genes. Particularly with regard to the diminished growth of PER-induced goiters, our data suggest that induction of PER-specific cellular stress responses might play an important role in the amphibian thyroid, including an up-regulation of negative growth regulators and de-regulation of genes with putative function in linking stress signalling and proliferative activity. Based on these findings, the important question arises whether the effects pattern observed in PER-treated tadpoles is primarily due to inhibition of iodide uptake, and thus represent a mode of action-specific response, or caused by increased intracellular levels of PER (Dohan et al., 2007). In order to identify expression profiles that are unique for certain modes of goitrogen action, further comparative analyses of expression profiles are currently performed for diverse inhibitors of NIS and TPO.

Recently, metamorphosis in anuran amphibians has been proposed as the most promising biological model for development of a non-mammalian bioassay to detect thyroid-disrupting xenobiotics (Helbing et al., 2007; Kloas, 2002; Opitz et al., 2006a). In this study, we identified an extended list of novel molecular biomarkers to detect altered thyroid function in amphibian tadpoles. Particularly, the temporal expression profiles determined in the present survey will aid in the design of short-term screening assays to assess chemicals for effects on thyroid function.

In conclusion, the present study highlights the value of amphibian metamorphosis in a novel non-mammalian model of thyroid goitrogenesis to improve our understanding of vertebrate thyroid function. By exploiting the high precision of real-time PCR, the gene expression profiling provides for the first time a list of molecular markers of altered thyroid function in a non-mammalian vertebrate. We have shown that expression profiles of key genes involved in TH synthesis and iodide metabolism are conserved between the amphibian and mammalian thyroid following goitrogen treatment. However, species-specific differences were also observed including unique expression patterns of *tshr* and iodothyronine deiodinases in the amphibian thyroid. Moreover, this amphibian model further allowed for the identification of thyroid responses that are common to goitrogens irrespective of their mode of action suggesting that TSH over-stimulation is the primary causative factor. On the other hand, this model generates strong evidence for the contention that additional factors, which might be related to the substance or its mode of action, contribute to the development of goiters with variable phenotypes. The expression data are also important because they have been obtained in developing larvae during a phase of late thyroid development. The list of candidate genes with altered expression in goitrous tadpole thyroids provides a valuable resource for further comparative studies addressing mechanistic aspects of thyroid gene expression regulation, developmental expression profiles, as well as the development of novel molecular biomarkers for detection of thyroid-disrupting xenobiotics in amphibian tadpoles.

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## References

- Adler, V., Yin, Z., Fuchs, S.Y., Benezra, M., Rosario, L., Tew, K.D., Pincus, M.R., Sardana, M., Henderson, C.J., Wolf, C.R., Davis, R.J., Ronai, Z., 1999. Regulation of JNK signaling by GSTp. *EMBO. J.* 18, 1321-1334.
- Arvan, P., Kim, P.S., Kuliawat, R., Prabakaran, D., Muresan, Z., Yoo, S.E., Abu, H.S., 1997. Intracellular protein transport to the thyrocyte plasma membrane: potential implications for thyroid physiology. *Thyroid.* 7, 89-105.
- Baris, O., Savagner, F., Nasser, V., Lorioud, B., Granjeaud, S., Guyetant, S., Franc, B., Rodien, P., Rohmer, V., Bertucci, F., Birnbaum, D., Malthiery, Y., Reynier, P., Houlgatte, R., 2004. Transcriptional profiling reveals coordinated up-regulation of oxidative metabolism genes in thyroid oncocytic tumors. *J. Clin. Endocrinol. Metab.* 89, 994-1005.
- Baryshev, M., Sargsyan, E., Wallin, G., Lejnicks, A., Furudate, S., Hishinuma, A., Mkrtchian, S., 2004. Unfolded protein response is involved in the pathology of human congenital hypothyroid goiter and rat non-goitrous congenital hypothyroidism. *J. Mol. Endocrinol.* 32, 903-920, 2004.
- Bidey, S.P., Hill, D.J., Eggo, M.C., 1999. Growth factors and goitrogenesis. *J. Endocrinol.* 160, 321-332.
- Bradford, C.M., Rinchar, J., Carr, J.A., Theodorakis, C., 2005. Perchlorate affects thyroid function in eastern mosquitofish (*Gambusia holbrooki*) at environmentally relevant concentrations. *Environ. Sci. Technol.* 39, 5190-5195.
- Bruhat, A., Averous, J., Carraro, V., Zhong, C., Reimold, A.M., Kilberg, M.S., Fafournoux, P., 2002. Differences in the molecular mechanisms involved in the transcriptional activation of the CHOP and asparagine synthetase genes in response to amino acid deprivation or activation of the unfolded protein response. *J. Biol. Chem.* 277, 48107-48114.
- Buckingham, M.B., 1970. Effects of TSH and thiourea upon thyroid function in the lizard, *Xantusia henshawi*. *Gen. Comp. Endocrinol.* 14, 178-183.
- Calebiro, D., de Filippis, T., Lucchi, S., Martinez, F., Porazzi, P., Trivellato, R., Locati, M., Beck-Peccoz, P., Persani, L., 2006. Selective modulation of protein kinase A I and II reveals distinct roles in thyroid cell gene expression and growth. *Mol. Endocrinol.* 20, 3196-3211.
- Coleman, R., Evannett, P.J., Dodd, J.M., 1967. The ultrastructural localization of acid phosphatase, alkaline phosphatase and adenosine triphosphatase in induced goitres of *Xenopus laevis* Daudin tadpoles. *Histochemie* 10, 33-43.
- Das, B., Cai, L., Carter, M.G., Piao, Y.L., Sharov, A.A., Ko, M.S., Brown, D.D., 2006. Gene expression changes at metamorphosis induced by thyroid hormone in *Xenopus laevis* tadpoles. *Dev. Biol.* 291, 342-355.
- De Felice, M., Postiglione, M.P., Di Lauro, R., 2004. Thyrotropin receptor signaling in development and differentiation of the thyroid gland: insights from mouse models and human diseases. *Endocrinology* 145, 4062-4067.
- Denef, J.F., 1989. Experimental goiter formation. *Bull Mem Acad R Med Belg* 144, 313-318.
- Denef, J.F., Haumont, S., Cornette, C., Beckers, C., 1981. Correlated functional and morphometric study of thyroid hyperplasia induced by iodine deficiency. *Endocrinology* 108, 2352-2358.

- Denver, R.J., Pavgi, S., Shi, Y.B., 1997. Thyroid hormone-dependent gene expression program for *Xenopus* neural development. *J. Biol. Chem.* 272, 8179-8188.
- Doerge, D.R., Takazawa, R.S., 1990. Mechanism of thyroid peroxidase inhibition by ethylenethiourea. *Chem. Res. Toxicol.* 3, 98-101.
- Dohan, O., Portulano, C., Basquin, C., Reyna-Neyra, A., Amzel, L.M., Carrasco, N., 2007. The Na<sup>+</sup>/I<sup>-</sup> symporter (NIS) mediates electroneutral active transport of the environmental pollutant perchlorate. *Proc. Natl. Acad. Sci. USA* 104, 20250-20255.
- Dorak, T., 2006. Real-Time PCR. *Advanced Methods Series*. Taylor & Francis, Oxford.
- Dumont, J.E., Lamy, F., Roger, P., Maenhaut, C., 1992. Physiological and pathological regulation of thyroid cell proliferation and differentiation by thyrotropin and other factors. *Physiol. Rev.* 72, 667-697.
- Dumont, J.E., Maenhaut, C., Lamy, F., 1992. Control of thyroid cell proliferation and goitrogenesis. *Trends Endocrinol. Metab.* 3, 12-17.
- Dunn, J.T., Dunn, A.D., 2001. Update on intrathyroidal iodine metabolism. *Thyroid.* 11, 407-414.
- Elsalini, O.A., von Gartzzen, J., Cramer, M., Rohr, K.B., 2003. Zebrafish *hhex*, *nk2.1a*, and *pax2.1* regulate thyroid growth and differentiation downstream of Nodal-dependent transcription factors. *Dev. Biol.* 263, 67-80.
- Eng, P.H., Cardona, G.R., Fang, S.L., Previti, M., Alex, S., Carrasco, N., Chin, W.W., Braverman, L.E., 1999. Escape from the acute Wolff-Chaikoff effect is associated with a decrease in thyroid sodium/iodide symporter messenger ribonucleic acid and protein. *Endocrinology* 140, 3404-3410.
- Furlow, J.D., Neff, E.S., 2006. A developmental switch induced by thyroid hormone: *Xenopus laevis* metamorphosis. *Trends Endocrinol. Metab.* 17, 40-47.
- Gereben, B., Salvatore, D., Harney, J.W., Tu, H.M., Larsen, P.R., 2001. The human, but not rat, *dio2* gene is stimulated by thyroid transcription factor-1 (TTF-1). *Mol. Endocrinol.* 15, 112-124.
- Glatt, C.M., Ouyang, M., Welsh, W., Green, J.W., Connor, J.O., Frame, S.R., Everds, N.E., Poindexter, G., Snajdr, S., Delker, D.A., 2005. Molecular characterization of thyroid toxicity: anchoring gene expression profiles to biochemical and pathologic end points. *Environ. Health Perspect.* 113, 1354-1361.
- Grommen, S.V., Taniuchi, S., Janssen, T., Schoofs, L., Takahashi, S., Takeuchi, S., Darras, V.M., De Groef, B., 2006. Molecular cloning, tissue distribution, and ontogenic thyroidal expression of the chicken thyrotropin receptor. *Endocrinology* 147, 3943-3951.
- Handa, R.J., Chiasson, R.B., 1980. Comparative effects of three goitrogenic treatments on White Leghorn chickens. *Avian Dis.* 24, 916-929.
- Helbing, C.C., Bailey, C.M., Ji, L., Gunderson, M.P., Zhang, F., Veldhoen, N., Skirrow, R.C., Mu, R., Lesperance, M., Holcombe, G.W., Kosian, P.A., Tietge, J., Korte, J.J., Degitz, S.J., 2007. Identification of gene expression indicators for thyroid axis disruption in a *Xenopus laevis* metamorphosis screening assay. Part 1. Effects on the brain. *Aquat. Toxicol.* 82, 227-241.
- Kameda, Y., 1984. Dog thyroid glands after chronic administration of antithyroid drugs. *Am. J. Pathol.* 117, 316-325.

- Kimura, T., Van Keymeulen, A., Golstein, J., Fusco, A., Dumont, J.E., Roger, P.P., 2001. Regulation of thyroid cell proliferation by TSH and other factors: a critical evaluation of in vitro models. *Endocr. Rev.* 22, 631-656.
- Kloas, W., 2002. Amphibians as a model for the study of endocrine disruptors. *Int. Rev. Cytol.* 216, 1-57.
- Kohrle, J., Jakob, F., Contempre, B., Dumont, J.E., 2005. Selenium, the thyroid, and the endocrine system. *Endocr. Rev.* 26, 944-984.
- Krause, K., Karger, S., Schierhorn, A., Poncin, S., Many, M.C., Fuhrer, D., 2007. Proteomic profiling of cold thyroid nodules. *Endocrinology* 148, 1754-1763.
- Krohn, K., Fuhrer, D., Bayer, Y., Eszlinger, M., Brauer, V., Neumann, S., Paschke, R., 2005. Molecular pathogenesis of euthyroid and toxic multinodular goiter. *Endocr. Rev.* 26, 504-524.
- Lalli, E., Sassone-Corsi, P., 1995. Thyroid-stimulating hormone (TSH)-directed induction of the CREM gene in the thyroid gland participates in the long-term desensitization of the TSH receptor. *Proc. Natl. Acad. Sci. USA* 92, 9633-9637.
- Leatherland, J.F., 1982. Environmental physiology of the teleostean thyroid gland: a review. *Env. Biol. Fish.* 7, 83-110.
- Lee, M.C., Miller, E.A., 2007. Molecular mechanisms of COPII vesicle formation. *Semin Cell Dev. Biol.* 18, 424-434.
- Lutz, I., Kloas, W., Springer, T.A., Holden, L.R., Wolf, J.C., Krueger, H.O., Hosmer, A.J., 2008. Development, standardization and refinement of procedures for evaluating effects of endocrine active compounds on development and sexual differentiation of *Xenopus laevis*. *Anal. Bioanal. Chem.* 390, 2031-2048.
- Maier, J., van Steeg, H., van Oostrom, C., Paschke, R., Weiss, R.E., Krohn, K., 2007. Iodine deficiency activates antioxidant genes and causes DNA damage in the thyroid gland of rats and mice. *Biochim. Biophys. Acta* 1773, 990-999.
- Manzon, R.G., Denver, R.J., 2004. Regulation of pituitary thyrotropin gene expression during *Xenopus* metamorphosis: negative feedback is functional throughout metamorphosis. *J. Endocrinol.* 182, 273-285.
- McNabb, F.M., 2007. The hypothalamic-pituitary-thyroid (HPT) axis in birds and its role in bird development and reproduction. *Crit. Rev. Toxicol.* 37, 163-193.
- Miranda, L.A., Pisano, A., Casco, V., 1996. Ultrastructural study on thyroid glands of *Bufo arenarum* larvae kept in potassium perchlorate solution. *Biocell* 20, 147-153.
- Nieuwkoop, P.D., Faber, J., 1994. Normal Table of *Xenopus laevis* (Daudin). Garland Publishing Inc., New York.
- Olen, E., 1969. The fine structure of experimentally induced hyperplastic and colloid goiter in the hamster. *Lab. Invest.* 21, 336-346.
- Opitz, R., Braunbeck, T., Bogi, C., Pickford, D.B., Nentwig, G., Oehlmann, J., Tooi, O., Lutz, I., Kloas, W., 2005. Description and initial evaluation of a *Xenopus* metamorphosis assay for detection of thyroid system-disrupting activities of environmental compounds. *Environ. Toxicol. Chem.* 24, 653-664.

- Opitz, R., Hartmann, S., Blank, T., Braunbeck, T., Lutz, I., Kloas, W., 2006a. Evaluation of histological and molecular endpoints for enhanced detection of thyroid system disruption in *Xenopus laevis* tadpoles. *Toxicol. Sci.* 90, 337-348.
- Opitz, R., Trubiroha, A., Lorenz, C., Lutz, I., Hartmann, S., Blank, T., Braunbeck, T., Kloas, W., 2006b. Expression of sodium-iodide symporter mRNA in the thyroid gland of *Xenopus laevis* tadpoles: developmental expression, effects of antithyroidal compounds, and regulation by TSH. *J. Endocrinol.* 190, 157-170, 2006.
- Ortiz-Caro, J., Pastor, R.M., Jolin, T., 1983. Effects of  $KClO_4$  in propylthiouracil-hypothyroid rats. *Acta Endocrinol.* 103, 81-87.
- Oyadomari, S., Mori, M., 2004. Roles of CHOP/GADD153 in endoplasmic reticulum stress. *Cell Death Differ.* 11, 381-389.
- Pedraza, P.E., Obregon, M.J., Escobar-Morreale, H.F., del Rey, F.E., de Escobar, G.M., 2006. Mechanisms of adaptation to iodine deficiency in rats: thyroid status is tissue specific. Its relevance for man. *Endocrinology* 147, 2098-2108.
- Pomerance, M., Carapau, D., Chantoux, F., Mockey, M., Correze, C., Francon, J., Blondeau, J.P., 2003. CCAAT/enhancer-binding protein-homologous protein expression and transcriptional activity are regulated by 3',5'-cyclic adenosine monophosphate in thyroid cells. *Mol. Endocrinol.* 17, 2283-2294.
- Regard, E., 1978. Cytophysiology of the amphibian thyroid gland through larval development and metamorphosis. *Int. Rev. Cytol.* 52, 81-118.
- Ron, D., Walter, P., 2007. Signal integration in the endoplasmic reticulum unfolded protein response. *Nat. Rev. Mol. Cell Biol.* 8, 519-529.
- Rusco, J.E., Rosario, L.A., Wang, T., Gate, L., Arifoglu, P., Wolf, C.R., Henderson, C.J., Ronai, Z., Tew, K.D., 2001. Pharmacologic or genetic manipulation of glutathione S-transferase P1-1 (GSTpi) influences cell proliferation pathways. *J. Pharmacol. Exp. Ther.* 298, 339-345.
- Sargsyan, E., Baryshev, M., Mkrtchian, S., 2004. The physiological unfolded protein response in the thyroid epithelial cells. *Biochem. Biophys. Res. Commun.* 322, 570-576.
- Sato, K., Yamazaki, K., Shizume, K., Kanaji, Y., Obara, T., Ohsumi, K., Demura, H., Yamaguchi, S., Shibuya, M., 1995. Stimulation by thyroid-stimulating hormone and Grave's immunoglobulin G of vascular endothelial growth factor mRNA expression in human thyroid follicles *in vitro* and flt mRNA expression in the rat thyroid *in vivo*. *J. Clin. Invest.* 96, 1295-1302.
- Scipioni, A., Ferretti, E., Soda, G., Tosi, E., Bruno, R., Costante, G., Meringolo, D., Arturi, F., Durante, C., Amorosi, A., Foschini, M.P., Nardi, F., Russo, D., Filetti, S., 2007. hNIS protein in thyroid: the iodine supply influences its expression and localization. *Thyroid.* 17, 613-618.
- Shi, Y.B., 1999. *Amphibian Metamorphosis. From Morphology to Molecular Biology.* John Wiley & Sons, New York.
- Srisomsap, C., Subhasitanont, P., Otto, A., Mueller, E.C., Punyarit, P., Wittmann-Liebold, B., Svasti, J., 2002. Detection of cathepsin B up-regulation in neoplastic thyroid tissues by proteomic analysis. *Proteomics* 2, 706-712.
- Stoker, T.E., Ferrell, J.M., Laws, S.C., Cooper, R.L., Buckalew, A., 2006. Evaluation of ammonium perchlorate in the endocrine disruptor screening and testing program's male pubertal protocol: ability to detect effects on thyroid endpoints. *Toxicology* 228, 58-65.

- Tietge, J.E., Holcombe, G.W., Flynn, K.M., Kosian, P.A., Korte, J.J., Anderson, L.E., Wolf, D.C., Degitz, S.J., 2005. Metamorphic inhibition of *Xenopus laevis* by sodium perchlorate: effects on development and thyroid histology. *Environ. Toxicol. Chem.* 24, 926-933.
- Uyttersprot, N., Pelgrims, N., Carrasco, N., Gervy, C., Maenhaut, C., Dumont, J.E., Miot, F., 1997. Moderate doses of iodide in vivo inhibit cell proliferation and the expression of thyroperoxidase and Na<sup>+</sup>/I<sup>-</sup> symporter mRNAs in dog thyroid. *Mol. Cell. Endocrinol.* 131, 195-203.
- Van der Geyten, S., Van den Eynde, I., Segers, I.B., Kuhn, E.R., Darras, V.M., 2002. Differential expression of iodothyronine deiodinases in chicken tissues during the last week of embryonic development. *Gen. Comp. Endocrinol.* 128, 65-73.
- van Staveren, W.C., Solis, D.W., Delys, L., Venet, D., Cappello, M., Andry, G., Dumont, J.E., Libert, F., Detours, V., Maenhaut, C., 2006a. Gene expression in human thyrocytes and autonomous adenomas reveals suppression of negative feedbacks in tumorigenesis. *Proc. Natl. Acad. Sci. USA* 103, 413-418.
- van Staveren, W.C., Detours, V., Dumont, J.E., Maenhaut, C., 2006b. Negative feedbacks in normal cell growth and their suppression in tumorigenesis. *Cell Cycle* 5, 571-572.
- Vassart, G., Dumont, J.E., 1992. The thyrotropin receptor and the regulation of thyrocyte function and growth. *Endocr. Rev.* 13, 596-611.
- Viglietto, G., Romano, A., Manzo, G., Chiappetta, G., Paoletti, I., Califano, D., Galati, M.G., Mauriello, V., Bruni, P., Lago, C.T., Fusco, A., Persico, M.G., 1997. Upregulation of the angiogenic factors PlGF, VEGF and their receptors (Flt-1, Flk-1/KDR) by TSH in cultured thyrocytes and in the thyroid gland of thiouracil-fed rats suggest a TSH-dependent paracrine mechanism for goiter hypervascularization. *Oncogene* 15, 2687-2698.
- Wolff, J., 1998. Perchlorate and the thyroid gland. *Pharmacol. Rev.* 50, 89-105.
- Wynford-Thomas, D., Stringer, B.M., Williams, E.D., 1982a. Desensitisation of rat thyroid to the growth-stimulating action of TSH during prolonged goitrogen administration. Persistence of refractoriness following withdrawal of stimulation. *Acta Endocrinol.* 101, 562-569.
- Wynford-Thomas, D., Stringer, B.M., Williams, E.D., 1982b. Goitrogen-induced thyroid growth in the rat: a quantitative morphometric study. *J. Endocrinol.* 94, 131-140.
- Xia, C., Hu, J., Ketterer, B., Taylor, J.B., 1996. The organization of the human GSTP1-1 gene promoter and its response to retinoic acid and cellular redox status. *Biochem. J.* 313, 155-161.
- Yamada, E., Yamazaki, K., Takano, K., Obara, T., Sato, K., 2006. Iodide inhibits vascular endothelial growth factor-A expression in cultured human thyroid follicles: a microarray search for effects of thyrotropin and iodide on angiogenesis factors. *Thyroid.* 16, 545-554.
- Yen, P.M., 2001. Physiological and molecular basis of thyroid hormone action. *Physiol. Rev.* 81, 1097-1142.
- Zambon, A.C., Zhang, L., Minovitsky, S., Kanter, J.R., Prabhakar, S., Salomonis, N., Vranizan, K., Dubchak, I., Conklin, B.R., Insel, P.A., 2005. Gene expression patterns define key transcriptional events in cell-cycle regulation by cAMP and protein kinase A. *Proc. Natl. Acad. Sci. USA* 102, 8561-8566.

**Fig. 1.** Temporal changes of developmental stages, whole body length (WBL) and hind limb length (HLL) during 12-day treatment of tadpoles with 20 mg/l perchlorate (PER) and 50 mg/l ethylenethiourea (ETU). Measurements were performed for 24 tadpoles per treatment on study day 1 and for 16 tadpoles per treatment on the remaining sampling dates. (A) Developmental stages were determined according to Nieuwkoop and Faber (1994), and median values are shown for each treatment group. Asterisks denote significant differences between treatment medians (\*\*\*)  $P < 0.001$ , Dunn's test). Results from WBL (B) and HLL (C) measurements are shown as means  $\pm$  SD. Asterisks denote significant differences between treatment means (\*\*\*)  $P < 0.001$ , Tukey-Kramer multiple comparison test).

**Fig. 2.** Relative expression levels of *thrb*, *bteb1-A*, *mcm2*, *pcna*, *kif2c* and *dapl1* mRNAs in tadpole brain after 8 days of treatment with 20 mg/l perchlorate (PER) and 50 mg/l ethylenethiourea (ETU). RNA was isolated from individual brain tissue samples and analyzed for relative mRNA levels of target transcripts by real-time PCR. *rpl8* mRNA was used for normalization of target gene expression. For graphical presentation, results for each target transcript were expressed relative to control group values (arbitrarily set at 1.0). Data are shown as means  $\pm$  SEM ( $n = 8$  for each treatment) and significant differences are marked by asterisks (\*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ , Tukey-Kramer multiple comparison test).

**Fig. 3.** Temporal changes of relative expression levels of *tshb-A* mRNA in tadpole pituitaries during 12-day treatment of tadpoles with 20 mg/l perchlorate (PER) and 50 mg/l ethylenethiourea (ETU). RNA was isolated from pituitary tissue sampled at the indicated time points and analyzed for relative mRNA levels of *tshb-A* by real-time PCR. *rpl8* mRNA was used for normalization of *tshb-A* expression. For graphical presentation, results were expressed relative to the control group values on study day 1 (arbitrarily set at 1.0). Data are shown as means  $\pm$  SEM ( $n = 8$  for each treatment). For each sampling date, significant differences between treatments are marked by asterisks (\*\*\*)  $P < 0.001$ , Tukey-Kramer multiple comparison test).

**Fig. 4.** Temporal changes in thyroid histology of *X. laevis* tadpoles during 12-day treatment of tadpoles with 20 mg/l perchlorate (PER) and 50 mg/l ethylenethiourea (ETU). In the control group (A-E), the progressive increase in glandular size during the experimental period was accompanied by increases in follicle number and in size of individual follicles. The colloid was PAS-positive with minimal peripheral vacuolation. Treatment with PER and ETU resulted in hyperplastic goiters with different follicular morphologies. In PER-treated tadpoles (F-J), collapsed follicles devoid of colloid and lined by high columnar epithelia predominated. Thyroids from ETU-treated tadpoles (K-O) displayed distended follicles with enlarged lumina containing

small amounts of foamy colloid. Sections of 3 - 4  $\mu\text{m}$  thickness were stained with periodic acid-Schiff (PAS) and Mayer's hematoxylin, and light micrographs were taken at 20 x magnification.

**Fig. 5.** Temporal changes of relative expression levels of *slc5a5*, *tpo* and *tshr* mRNAs in tadpole thyroids during 12-day treatment of tadpoles with 20 mg/l perchlorate (PER) and 50 mg/l ethylenethiourea (ETU). RNA was isolated from thyroid tissue sampled at the indicated time points and analyzed for relative mRNA levels of target transcripts by real-time PCR. *eef1a* mRNA was used for normalization of target gene expression. For graphical presentation, results were expressed relative to the control group values on study day 1 (arbitrarily set at 1.0). Data are shown as means  $\pm$  SEM ( $n = 8$  for each treatment). For each sampling date, significant differences between treatments are marked by asterisks (\*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ , Dunn's test for *slc5a5* data and Tukey-Kramer multiple comparison test for *tpo* and *tshr* data). The target transcript is indicated for each graph.

**Fig. 6.** Temporal changes of relative expression levels of *eif4a1*, *hspa5*, *sar1a* and *rnp24* mRNAs in tadpole thyroids during 12-day treatment of tadpoles with 20 mg/l perchlorate (PER) and 50 mg/l ethylenethiourea (ETU). RNA was isolated from thyroid tissue sampled at the indicated time points and analyzed for relative mRNA levels of target transcripts by real-time PCR. *eef1a* mRNA was used for normalization of target gene expression. For graphical presentation, results were expressed relative to the control group values on study day 1 (arbitrarily set at 1.0). Data are shown as means  $\pm$  SEM ( $n = 8$  for each treatment). For each sampling date, significant differences between treatments are marked by asterisks (\*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ , Dunn's test for *sar1a* and *hsp5a* data and Tukey-Kramer multiple comparison test for *eif4a1* and *rnp24* data). The target transcript is indicated for each graph.

**Fig. 7.** Temporal changes of relative expression levels of *mcm2*, *pcna*, *vegf-A* and *tie-2* mRNAs in tadpole thyroids during 12-day treatment of tadpoles with 20 mg/l perchlorate (PER) and 50 mg/l ethylenethiourea (ETU). RNA was isolated from thyroid tissue sampled at the indicated time points and analyzed for relative mRNA levels of target transcripts by real-time PCR. *eef1a* mRNA was used for normalization of target gene expression. For graphical presentation, results were expressed relative to the control group values on study day 1 (arbitrarily set at 1.0). Data are shown as means  $\pm$  SEM ( $n = 8$  for each treatment). For each sampling date, significant differences between treatments are marked by asterisks (\*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ , Tukey-Kramer multiple comparison test). The target transcript is indicated for each graph.

**Fig. 8.** Temporal changes of relative expression levels of *gadd153*, *asns* and *gstp1* mRNAs in tadpole thyroids during 12-day treatment of tadpoles with 20 mg/l perchlorate (PER) and 50 mg/l ethylenethiourea (ETU). RNA was isolated from thyroid tissue sampled at the indicated time points and analyzed for relative mRNA levels of target transcripts by real-time PCR. *eef1a* mRNA was used for normalization of target gene expression. For graphical presentation, results were expressed relative to the control group values on study day 1 (arbitrarily set at 1.0). Data are shown as means  $\pm$  SEM ( $n = 8$  for each treatment). For each sampling date, significant differences between treatments are marked by asterisks (\*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ , Dunn's test). The target transcript is indicated for each graph.

**Table 1. List of candidate genes analyzed for relative expression levels in thyroid tissue sampled on study day 12<sup>a</sup>**

NCBI		relative expression <sup>b</sup>		
accession	Gene description	PER/Co	ETU/Co	PER/ETU
<b>TH synthesis, secretion and metabolism</b>				
BC077614	slc5a5, solute carrier transporter 5a5 [sodium-iodide symporter, NIS]	62.56 *	32.28 *	1.94 *
AM406797	tpo, thyroid peroxidase	4.15 *	3.71 *	1.12
EB738209	tshr, thyroid-stimulating hormone receptor	3.27 *	3.15 *	1.04
Ensembl <sup>c</sup>	tg, thyroglobulin, predicted	1.84 *	1.88 *	0.98
DQ098656	dio1, iodothyronine type I deiodinase	7.29 *	1.39	5.25 *
AF354707	dio2, iodothyronine type II deiodinase	5.70 *	5.21 *	1.09
BC106400	dio3, iodothyronine type III deiodinase	0.01 *	0.02 *	0.53
BC078565	dehal1, iodothyronine dehalogenase	3.00 *	2.42 *	1.24
BC046667	ctsb, cathepsin B	2.57 *	2.09 *	1.23
<b>RNA translation</b>				
Z14253	etf1, eukaryotic translation termination factor 1	3.69 *	3.77 *	0.98
BC106279	eif4a1, eukaryotic translation initiation factor 4A1	2.54 *	2.62 *	0.97
D31837	eif4e, eukaryotic translation initiation factor 4E	1.92 *	1.81 *	1.06
L37045	gspt1, G1 to S phase transition 1	2.36 *	2.76 *	0.86
BC077825	gspt2, G1 to S phase transition 2	2.31 *	2.31 *	1.00
X88927	nolc1, nucleolar and coiled-body phosphoprotein 1	1.80 *	2.07 *	0.87
<b>protein folding and endoplasmic reticulum function</b>				
BC041192	hspd1, heat shock 60kDa protein 1 [aka chaperonin]	4.80 *	3.66 *	1.31
BC077757	hspa5, heavy-chain binding protein [aka BIP]	3.49 *	3.74 *	0.93
BC077228	pdia6, protein disulfide isomerase-related protein	3.06 *	3.15 *	0.97
BC043852	xbp1, X-box binding protein 1	2.43 *	2.08 *	1.17
BC077754	ero1l, endoplasmic reticulum oxidoreductin 1-like ( <i>S. cerevisiae</i> )	2.25 *	2.51 *	0.90
BC134823	edem1, ER degradation enhancer, mannosidase alpha-like 1	2.08 *	2.70 *	0.77
<b>vesicular protein transport</b>				
BC061656	sar1a, sar1a protein	14.48 *	17.59 *	0.82
BC044095	rnp24, coated vesicle membrane protein rnp24	2.62 *	3.16 *	0.83
BC043866	rab5a, member RAS oncogene family	2.61 *	2.24 *	1.17
BC077884	rab7, member RAS oncogene family	2.11 *	2.12 *	0.99
BC082352	sec24c, SEC24-related protein C	2.01 *	2.36 *	0.85
BC075251	copa, coatamer protein complex, subunit alpha	1.55 *	1.43 *	1.08
<b>cell growth and proliferation</b>				
M14455	myc, similar to myelocytomatosis oncogene, c-myc I	0.63	0.72	0.87
BC070712	myc, proto-oncogene c-myc II	1.18	1.24	0.95
M34080	pna, proliferating cell nuclear antigen	0.86	1.41 *	0.61 *
D63919	mcm2, minichromosome maintenance protein 2	0.86	1.53 *	0.56 *
U44048	mcm5, minichromosome maintenance protein 5	0.60 *	1.12	0.53 *
BC041525	ccnd1, cyclin D1	0.70 *	1.20	0.58 *
BC041288	aurkb, aurora kinase B	0.85	1.62 *	0.52 *
BC044976	kif2c, kinesin family member 2C	1.24	1.72 *	0.72
D00679	chc1, chromosome condensation 1	1.33	1.50 *	0.89
M29857	igf1, insulin-like growth factor 1	n.d.	n.d.	n.d.
AY050645	igf2, insulin-like growth factor 2	2.02 *	1.90 *	1.06
<b>negative growth regulation, growth arrest and apoptosis</b>				
AY302440	gadd153, transcription factor gadd153 [aka CHOP]	5.98 *	0.80	7.46 *
BC044046	gadd45a, growth arrest and DNA-damage-inducible, alpha	2.06 *	2.12 *	0.97
AJ141384	gadd45g, growth arrest and DNA-damage-inducible, gamma	32.24 *	7.00 *	4.61 *
AB048259	sesn1, sestrin 1	1.73 *	1.08	1.60 *
BC110790	bcl-xl, bcl-XL protein	1.85 *	2.13 *	0.87
BX854692	bag1, bcl2-associated athanogene-1	1.79 *	1.88 *	0.95
BC133188	dapl1, death-associated protein-like 1	0.55 *	0.48 *	1.15
BC076835	gas6, growth arrest-specific 6	0.21 *	0.31 *	0.67
<b>stress response</b>				
BC084983	LOC495456, similar to protein phosphatase 1, regulatory subunit 15B	2.76 *	1.93 *	1.42 *
BC043997	nrf2, nuclear factor (erythroid-derived 2)-like 2	2.27 *	1.89 *	1.20
AY362041	sod2, manganese superoxide dismutase 2	2.19 *	1.77 *	1.24
BC054964	cat, catalase	1.54 *	1.51 *	1.02
BC043769	hif1a, hypoxia-inducible factor 1, alpha subunit	1.84 *	1.53 *	1.20

BC054171	gstm2, glutathione S-transferase, mu 2	1.86 *	1.86 *	1.00
AJ489617	gstp1, glutathione S-transferase, pi	0.38 *	5.79 *	0.07 *
<b>angiogenesis</b>				
AF008593	vegfa, vascular endothelial growth factor A	1.29	1.17	1.10
AF007760	kdr-a, vegf receptor 2	1.24	1.08	1.15
AY037939	tie-2, endothelium-specific receptor tyrosine kinase 2	0.77 *	0.56 *	1.38
BC073367	ang1, angiopoietin-1	0.69 *	0.80	0.86
<b>miscellaneous</b>				
BC075318	asns, asparagine synthetase	12.45 *	1.71	7.28 *
BC044004	odc-1, ornithine decarboxylase 1	1.41 *	1.41 *	1.00
BC077402	cldn6, claudin-6	0.12 *	0.10 *	1.23

<sup>a</sup> RNA was isolated from eight individual thyroid tissue samples per treatment and analyzed for relative mRNA levels of target transcripts by real-time PCR. Elongation factor 1 $\alpha$  mRNA was used for normalization of target gene expression.

<sup>b</sup> Reported values for relative expression are ratios between the mean expression value of the PER treatment and the control (PER/Co), the ETU treatment and the control (ETU/Co), and the PER treatment and the ETU treatment (PER/ETU).

<sup>c</sup> Transcript information were derived from *Xenopus tropicalis* genome assembly version 3 ([http://www.ensembl.org/Xenopus\\_tropicalis/index.html](http://www.ensembl.org/Xenopus_tropicalis/index.html))

Asterisks denote significant differences between treatments ( $P < 0.05$ , Tukey-Kramer multiple comparison test or Dunn's test). n.d. Relative expression values were not calculated because igf-1 mRNA expression was detectable but below the limit of quantification ( $C_T > 35$ ) in the PER treatment group and was not detectable ( $C_T > 40$ ) in 6/8 samples of the ETU treatment group.

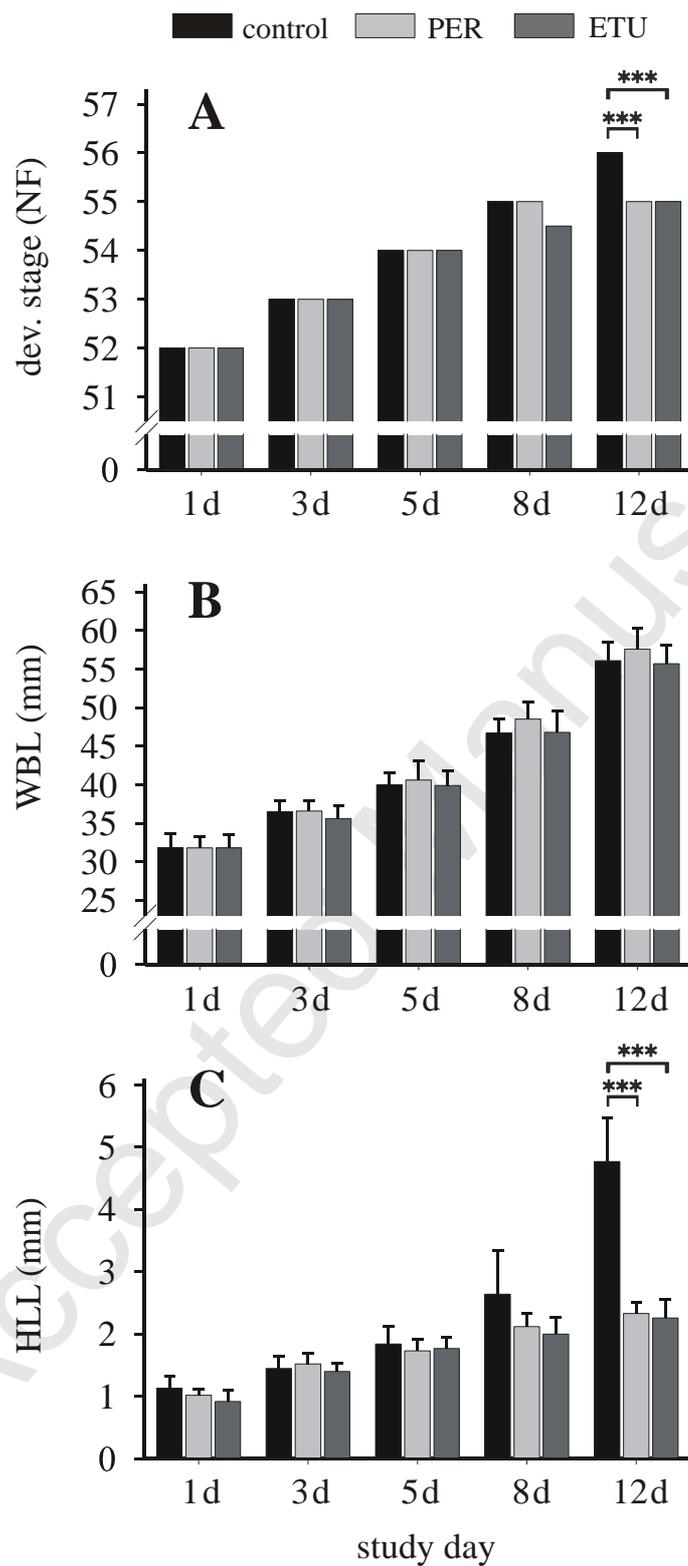
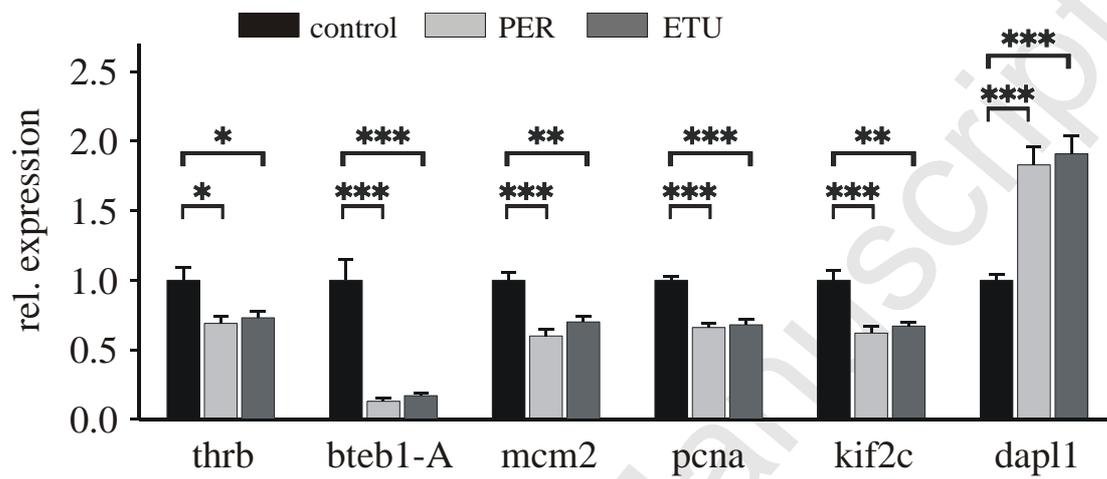
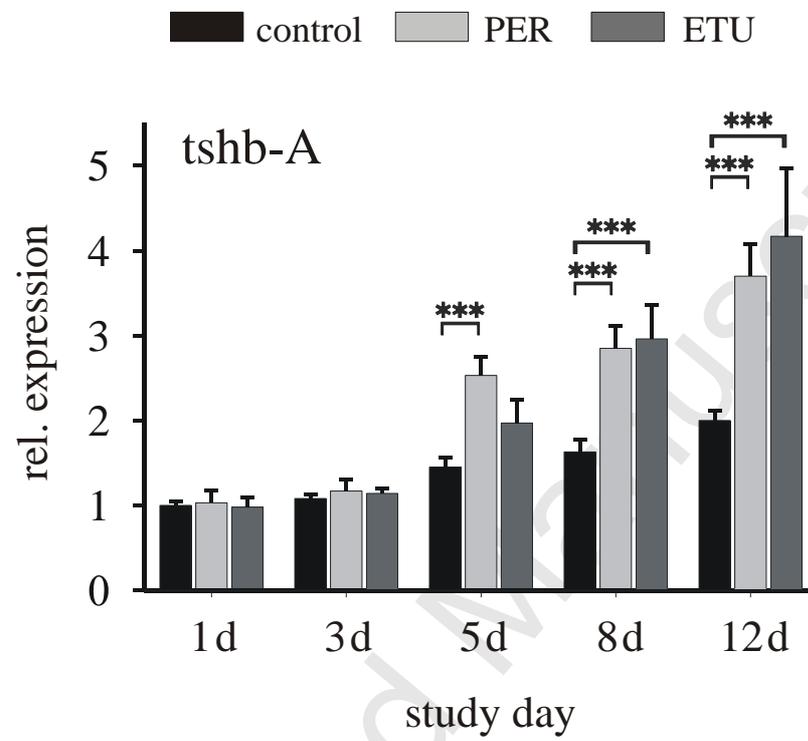
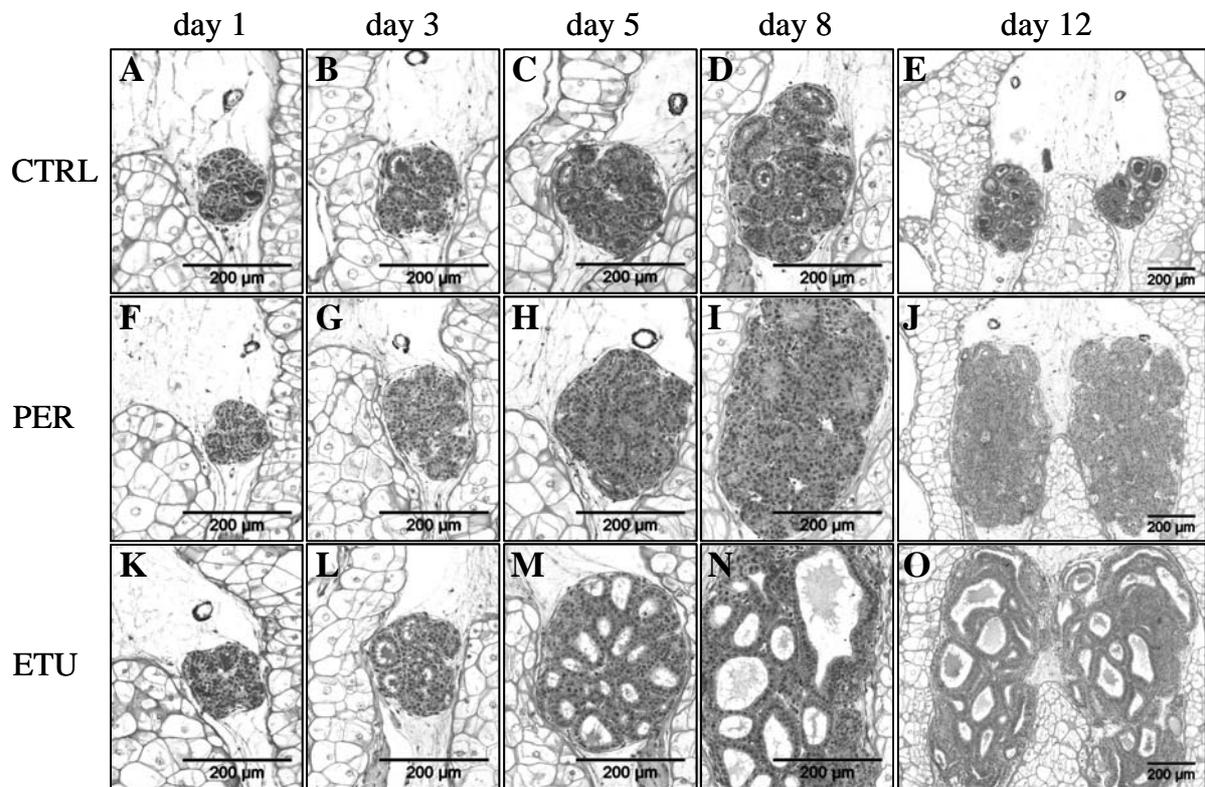
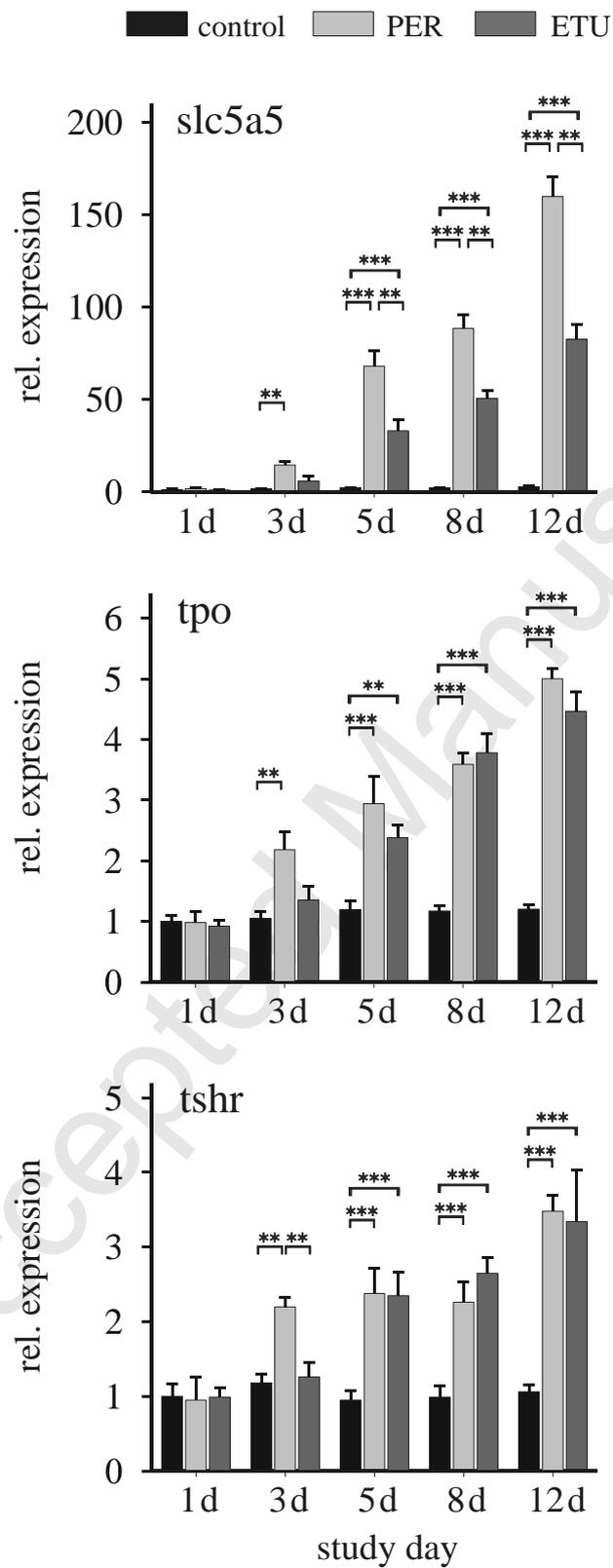


Figure 1

**Figure-2**

**Figure-3**

**Figure-4**

**Figure-5**

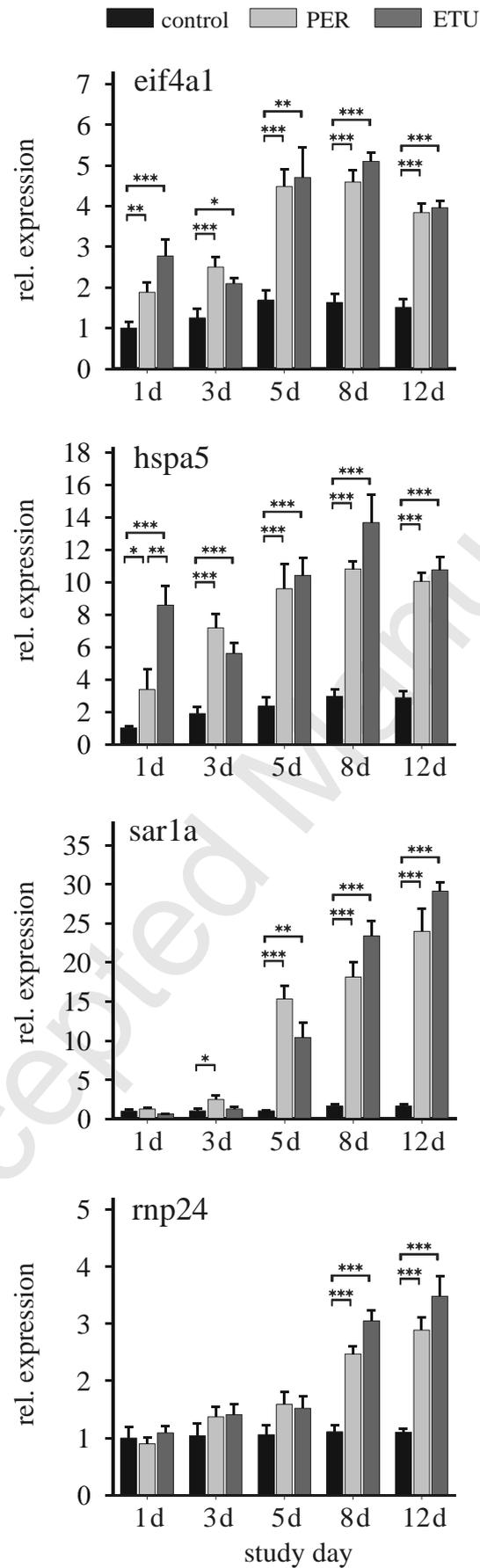


Figure-6

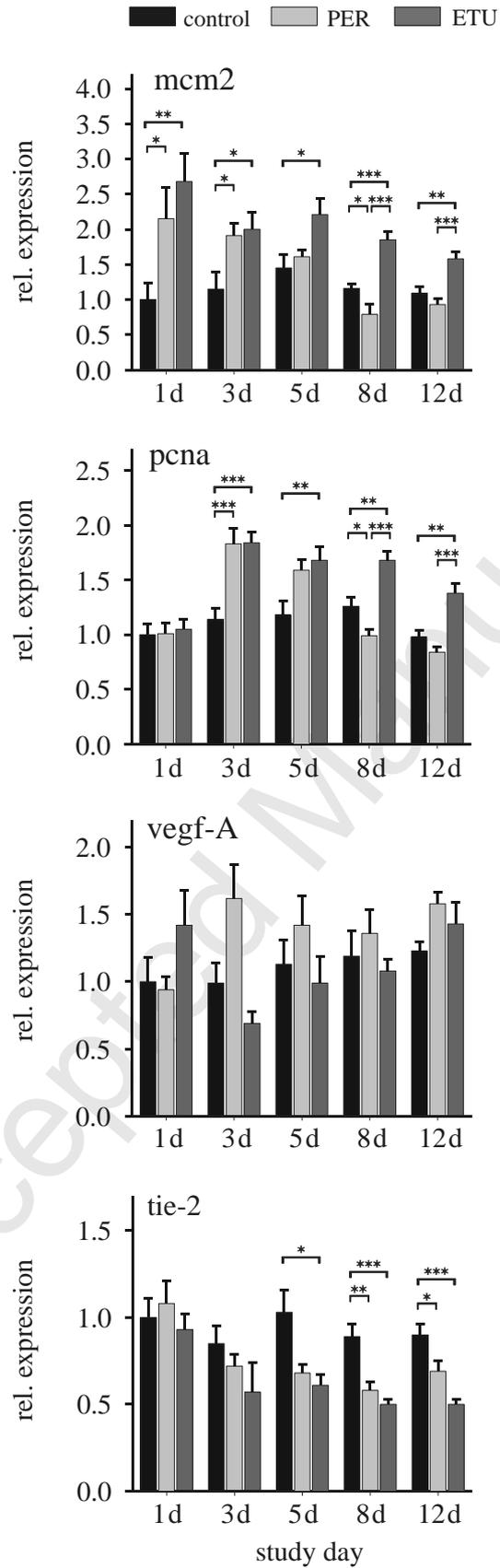


Figure-7

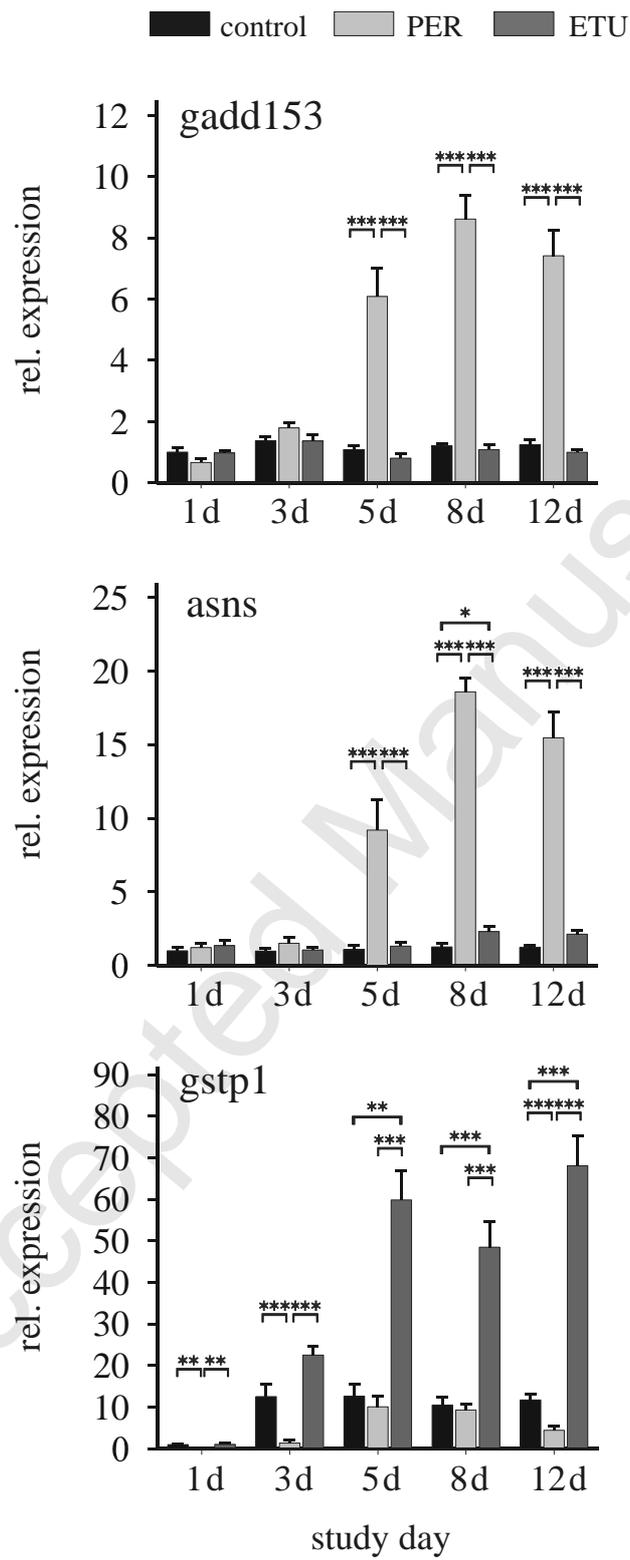


Figure-8