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Pascal GP Martin[1], Véronique Dupouy[1,2], Julien Leghait[1], Thierry Pineau[1], Arnaud Polizzi[1], Frédéric Lasserre[1], Béatrice B. Roques[1,2], Catherine Viguié[1*]

[1] ToxAlim (Research Centre in Food Toxicology), Université de Toulouse, UMR1331 INRA, ENVT, INP-Purpan, UPS, 180 chemin de Tournefeuille, BP93173, F-31027 Toulouse Cedex 03, France

[2] Current address: IntheRes, UMR1436 INRA, ENVT, Ecole Nationale Vétérinaire de Toulouse, 23 chemin des Capelles, BP87614, F-31076 Toulouse Cedex 03, France

* Corresponding author : Catherine Viguié, catherine.viguie@inra.fr

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ABSTRACT

Fipronil is a phenylpyrazole insecticide used for the control of a variety of pest for domestic, veterinary and agricultural uses. Fipronil exposure is associated to thyroid disruption in the rat. It increases thyroid hormone (TH) hepatic clearance. The effect on thyroxine (T4) clearance is about four fold higher than the effect on T4 plasma concentrations suggesting that the thyroid gland might develop compensatory mechanisms. The aim of this study was to document the potential effects of fipronil treatment on the thyroid transcriptome together with its effects on TSH and TH blood levels under well characterized internal exposure to fipronil and its main metabolite fipronil sulfone.

Fipronil (3 mg/kg/d by gavage for 14 days) clearance increased while its half-life decreased (about 10 fold) throughout treatment. Fipronil treatment in adult female rats significantly decreased total T4 and free triiodothyronine (T3) concentrations. Key genes related to thyroid hormone synthesis and/or cellular dynamic were modulated by fipronil exposure. RT-PCR confirmed that thyroglobulin gene expression was upregulated. A trend toward higher Na/I symporter expression was also noted, while sulfotransferase 1a1 gene expression was down-regulated. The expression of genes potentially involved in thyroid cell dynamic were upregulated (e.g. prostaglandin synthase 1, amphiregulin and Rhoa). Our results indicate that both pathways of TH synthesis and thyroid cell dynamics are transcriptional targets of fipronil and/or its main sulfone metabolite. The underlying mechanisms remain to be elucidated.

Key-words: Thyroid- Fipronil- Toxicokinetic- Endocrine disruptor

1. Introduction

Fipronil is a broad-spectrum phenylpyrazole insecticide that is extensively used for the control of a variety of pest. In particular, it is commonly used in veterinary medicine as a topical insecticide and acaricide for flea and tick control in dogs and cats, but also as a domestic biocide and, in some countries, as an agronomic insecticide. From its uses, fipronil can constitute a contaminant of the domestic environment (Mahler *et al.*, 2009; Lee *et al.*, 2010) and/or a food pollutant (Le Faouder *et*

al., 2007; Doran *et al.*, 2008) with a potential wide exposure of human populations in which fetal exposure to fipronil main metabolite, fipronil sulfone has been described (Kim *et al.*, 2019). Interestingly, in this latter study fipronil sulfone cord blood concentration was inversely correlated to those of total and free T3 as well as 5-min Apgar scores of newborn infants. Within the context of occupational exposure to fipronil (workers in a plant conditioning fipronil), fipronil sulfone concentrations were negatively correlated with serum TSH concentrations in fipronil-exposed workers, raising the possibility that fipronil has a central inhibitory effect on TSH secretion in humans (Herin *et al.*, 2011).

From a safety point of view, fipronil is classified by the World Health Organization as a Class II moderately hazardous pesticide (WHO, 2009). The U.S. EPA has classified fipronil as "Group C - possible human carcinogen" based on increases in thyroid follicular tumors in the rat" (U.S. Environmental Protection Agency, 2000). In its scientific reports of regulatory toxicology evaluation, EFSA concluded that fipronil did not exhibit genotoxicity and that thyroid tumors in the rat were not relevant to human. However, fipronil proved to be mutagenic, recombinogenic and carcinogenic in a model of somatic cells of Drosophila melanogaster (de Morais *et al.*, 2016) and genotoxic in an automated detection system of γH2AX in the human hepatic cell line HepaRG at a concentration of 15 µM, far below the cytotoxic IC 50 of 118µM, for 7 days exposure (Quesnot *et al.*, 2016). The acknowledged NOAEL is 0.019 mg/kg/day leading to a RfD of 0.2 µg/kg/day (EFSA, 2006).

In rat, fipronil and/or fipronil sulfone-treatment induces hepatic enzymatic activities leading to increased elimination of thyroid hormones (Leghait *et al.*, 2009; Roques *et al.*, 2012; Roques *et al.*, 2013). This effect can be evidenced on both total and free thyroxine clearances and results in a decrease in circulating thyroid hormone (TH) (Leghait *et al.*, 2009; Moser *et al.*, 2015) concentrations together with increased thyroid-stimulating hormone (TSH) plasma concentration. It is noteworthy however that the effect of fipronil and/or its main metabolite fipronil sulfone on L-thyroxine (T4) clearance (up to 100% increase) is much higher than their effects on circulating T4 (25% decrease at the most). It is likely that the thyroid gland develops compensatory mechanisms allowing to overcome, at least in part, the increased TH elimination induced by fipronil as a result of activation of hepatic

metabolism (Roques *et al.*, 2013). The deleterious consequences of fipronil-induced TH hepatic catabolism might thus be dependent upon the compensatory capacities of the thyroid gland. Evaluating the importance of compensatory mechanisms and whether those mechanisms can lead to deleterious effects on the thyroid gland physiology is thus an important issue with regard to hazard assessment of fipronil.

The increased TSH concentrations observed in rats exposed to fipronil (Leghait *et al.*, 2009), can be explained by a reduced negative feedback of TH at the hypothalamo-pituitary level. Many steps of TH biosynthesis and secretion are under the control of TSH. In particular, the two key and limiting steps of their biosynthesis, namely iodine supply to the thyreocytes and the iodine organification resulting in the iodination of tyrosine residues of the thyroglobulin (TG) protein. Indeed, thyroperoxidase (TPO), the key enzyme in iodine utilization, and the sodium-iodide symporter (NIS) gene expressions are both positively regulated by TSH (Pratt *et al.*, 1989; Kogai *et al.*, 1997). Furthermore, TSH can impact the thyroid gland cellular dynamic (Thomas and Williams, 1999). As a consequence, high TSH levels are acknowledged in rodents as a determining factor for the development of thyroid cancer (McClain, 1989; McClain, 1992; McClain, 1995). Long term fipronil treatment has been shown to be associated with numerous alterations of the thyroid histology in mouse (Ferreira *et al.*, 2012; Rodrigues da Cunha E. *et al.*, 2017) and to a higher incidence of thyroid tumours along with increased TSH in rats in pharmacological regulatory surveys (U.S. Environmental Protection Agency, 2000; EFSA, 2006).

To date, there is no data on gene expression changes in the thyroid gland upon fipronil exposure that might explain the changes induced by fipronil in thyroid function and thyroid gland histology. In addition, pharmacological non peer-reviewed (U.S. Environmental Protection Agency, 2000; EFSA, 2006) and laboratory (Cravedi *et al.*, 2013) studies indicate that fipronil and/or its metabolites can be found in the thyroid gland. There is no information available concerning the functional significance of such a contamination of the gland and whether fipronil can directly alter molecular pathways within the thyroid gland.

The goal of this study was to provide insights for a better understanding of the pathophysiological changes resulting from fipronil exposure, at the level of the thyroid gland itself.

We assessed genome-wide gene expression changes within the thyroid gland under well-characterized internal exposure to fipronil and its main metabolite fipronil sulfone.

2. Materials and methods

2.1. Test material, chemicals and fipronil treatment

Chemicals were purchased from Sigma Aldrich (Saint-Quentin Fallavier, France) unless otherwise specified. A phytosanitary-grade fipronil was used as more relevant to "field exposure" than standard-grade" fipronil. Fipronil (lot N° B20050318, purity 95.6%) was purchased from 3B Medical Systems Inc. (Libertyville, IL, USA). The contaminants as identified by HPLC/UV detection were mainly fipronil sulfone, the main metabolite of fipronil formed *in vivo* and with similar effects on the thyroid homeostasis than fipronil. Fipronil treatment consisted of a fipronil suspension. The vehicle was an aqueous methyl cellulose (0.5% w/w) and Tween 80 (0.01% w/v) solution. The fipronil suspension (1.5 mg/ml) was kept protected from light and was stirred before each administration. The rats received a daily administration of vehicle or fipronil (3 mg/kg/d) by gavage for 14 days.

2.2. Animal housing

All experiments were performed on adult female Wistar rats. For the preliminary experiment, the rats came from the INRA UMR 1331 inbred colony. For the main experiment, the rats were Wistar HsdHan: WIST Outbred from Charles River Laboratories, France. All animals were maintained at room temperature with a reversed 12h light/dark cycle. They were acclimatised for at least two weeks before the beginning of the experiments. For the preliminary experiment, the rats were housed one per cage after catheterization of the femoral vein. For the main experiment, the rats were housed 4 per cage throughout the experiment. The rats had free access to food (2016 Teklad Diet, Harlan, Gannat, France) and tap water and were weighed twice a week. All animal procedures were carried out in accordance with the accepted standards of humane animal care under the agreement number 31-242 for animal experimentation from the French Ministry of Agriculture

2.3. Preliminary experiment: characterization of fipronil toxicokinetic under repeated oral administrations

This experiment was performed on three 6 month-old female Wistar rats (mean body weight (±SD): 280 ± 13 g). One month before the kinetic investigations, catheters were surgically inserted into one femoral vein under anaesthesia with ketamine (50 mg/kg) / medetomidine (0.2 mg/kg) ip (Imalgene®1000, Merial SAS, Villeurbanne, France/Domitor®, Pfizer, Paris, France). During the post-surgical stage, rats received daily subcutaneous (sc) injections of an anti-infectious drug (sulfadoxine (7.5 mg/kg) and trimethoprime (1.5 mg/kg); Borgal®, Intervet SA, Angers, France) and an anti-inflammatory and antalgic treatment (flunixine meglumine 5 mg/kg/d; Meflosyl®) for 2 days.

The rats received a daily gastric administration of fipronil (3mg/kg/d) for 14 days as described above. Rats were weighted every 5-6 days and the volume of injection was adjusted to the most recently recorded bodyweight. Blood was collected in heparinized tubes 2, 5, 8 and 24h after the first, and just before the fourth and the eleventh administrations, and 2, 5, 8, 24 and 100 h after the last administration in order to characterize the time course of plasma fipronil and fipronil sulfone concentrations during repeated oral administrations.

After each blood sample (200 µL), a volume of physiological saline equivalent to the collected blood volume was administered, followed by 150 µL of heparinized saline (Heparine choay®, 50 UI/mL, Sanofi-aventis, France). Blood samples were centrifuged at 4000 g for 15 min at 4°C and the plasma stored at -20°C until assay.

2.4. Main experiment: effect of fipronil treatment on thyroid hormone secretion and gene expression within the thyroid gland

The experiment was performed on 24 adult female Wistar rats (mean body weight (±SD): 202.4 ± 6 g). They were randomly allocated to two groups: fipronil (3 mg/kg/day, n=10) and vehicle-treated (n=14) groups. The four rats with the lowest thyroid RNA quality in the control group were excluded from the thyroid transcriptomic study. TH and TSH data for all the 24 animals are reported elsewhere (Roques *et al.*, 2013). Here, we only report TH and TSH levels for those animals for which thyroid mRNA was used for microarray and qPCR analyses (n=10 vehicle; n=10 fipronil).

155 On average, twenty five hours after the last fipronil administration, rats were euthanized by
156 CO_2 inhalation. Total blood was collected by post-mortem puncture of the posterior vena cava for
157 hormone and toxicant assays. Blood was centrifuged at 3000 g for 10 min at 4 °C and plasma decanted
158 and kept frozen at -20°C until assays. The thyroid gland was rapidly dissected, snap frozen in liquid
159 nitrogen and kept at -80°C until RNA extraction.
160
161 **2.5. TSH and Thyroid hormone assays**
162 Total and free T4 and T3 plasma concentrations were determined using radioimmunoassay kits from
163 Diagnostic Products Corporation (Los Angeles, CA, USA) previously validated for assay in rat
164 (Leghait *et al.*, 2009). TSH plasma concentrations were measured by RIA kit (Biocode Hycel, Massy,
165 France). All assays were performed according to the manufacturer's instructions in one run for each
166 hormone. The mean intra assay for 3 quality control (QC) pools were less than 13% for total and free
167 T4, total and free T3 and TSH concentrations. The limit of quantification of the total T4 assay was
168 validate at 5 ng/ml. The limit of detection for the other assays was set at the lowest value of the
169 standard curve *i.e*. 1.1 pg/ml, 0.2 ng/ml, 0.52 pg/ml and 2.1 ng/ml for free T4, total T3, free T3 and
170 TSH, respectively.
171
172 **2.6. Fipronil and fipronil sulfone assay**
173 For the preliminary experiment, the measurement of plasma fipronil and fipronil sulfone
174 concentrations were done by Amatsigroup laboratories (Fontenilles, France) by HPLC/MS/MS from
175 50 µL of plasma after liquid/liquid extraction. An Alliance 2695 chromatographic system coupled with
176 a triple quadrupole mass spectrometer Quattro Micro™ API (Waters Corporation, Saint Quentin en
177 Yvelines, France). The analytes were separated on a C18 column (BDS Hypersil C18, 3 µm; 100 x 3
178 mm, Thermo Electron Corporation, Courtabœuf, France). The mass spectrometry detection was done
179 with the MRM (Multiple Reaction Monitoring) mode with a negative electrospray ionisation. The
180 MRM transitions monitored were m/z: 435 > 330 and m/z: 451 > 415, for fipronil and fipronil sulfone
181 analysis, respectively, with collision energies of 16 and 17 eV. Three quality control (QC) pools of 8,
182 30 and 80 ng/mL were used for the validation run. The mean within-day precision for the three QC

was 3.2% for fipronil and 6.9% for fipronil sulfone. The accuracy was 96 ± 10.21% for fipronil and 108 ± 15.05% for fipronil sulfone. The limit of quantification (LOQ) was 5 ng/mL for both fipronil and fipronil sulfone.

For the main experiment, fipronil and fipronil sulfone plasma concentrations were determined by high-performance liquid chromatography (HPLC) coupled with an ultraviolet and mass spectrometry (UV/MS) detection method as previously described in a single assay (Lacroix *et al.*, 2010). The mean within day precision was lower than 12% for both molecules. The limit of quantification of the assay was 2.5 ng/ml for both molecules.

2.7. Pharmacokinetic analysis

The pharmacokinetic analyses were performed by least-squares regression analysis using WinNonlin® software as previously described (Leghait *et al.*, 2009). The time course of fipronil concentrations were fitted using a monoexponential equation with a first-order absorption phase and clearance defined as a time-dependent variable to account for by the dramatic increase in fipronil elimination rate between the first and the last administrations. The data were weighted by the inverse of the fitted values. The terminal half-life ($t_{1/2}$), the time (T_{max}) at the maximal concentration (C_{max}), the area under the concentration curve *vs.* time (AUC) from time zero to the last detectable concentration and the apparent clearance were determined.

2.8. Transcriptome analysis by microarray and qPCR

Total RNA was extracted from frozen thyroid glands using Rneasy mini kits plus (Qiagen, Courtabœuf, France) according to the manufacturer's instruction. The extracted RNA samples were controlled for integrity on an Agilent 2100 Bioanalyzer (Agilent Technologies, Massy, France) and assayed at 260 nm. Thyroid gene expression profiles were determined using a whole rat genome microarray (4 x 44 K) from Agilent Technologies (Les Ulis, France). Labelled cRNA were prepared from the purified RNA samples using the fluorescent probes cyanine 3-CTP (Cy3) or cyanine-5CTP (Cy5) according to Agilent's protocol. For each group, the RNA samples from 5 animals were labelled

with Cy5 and the other 5 were labelled with Cy3. Samples were hybridized competitively on 10 microarrays using a dye-switch design (one Cy3-labelled sample from one experimental group vs one Cy5-labelled sample from the other group on each microarray). After completing the hybridization procedure, microarrays were scanned on a Genepix 4000B® scanner and signal was quantified using Agilent Feature Extraction Software v9.5. The raw and processed data, together with details of the experimental procedure and data analysis are available in the Gene Expression Omnibus (GO) database under the accession number GSE75275.

All details of microarray data filtering, normalization and quality controls are described in GSE75275. Data were analysed under R (v. 2.6.1, www.r-project.org) using packages from Bioconductor (www.bioconductor.org). Briefly, only spots passing a series of quality controls on 8 out of 10 microarrays were analysed and replicated probes were summarized by their median intensity. Data were normalized within (loess) and between arrays (Aquantile) using R limma package. Microarray data were analysed for gene expression changes using a separate channel analysis as described in limma user guide and GSE75275. We selected 228 probes corresponding to differentially expressed genes (raw $p<0.005$ and q-value <0.39), used hierarchical clustering to represent the data as a heatmap and analysed the enrichment of Gene Ontology biological processes among the upregulated and downregulated genes using the GOstats R package.

qPCR RNA samples (2µg) were converted to cDNA using the High Capacity Reverse Transcription kit (Applied Biosystems). Real time qPCR amplifications were performed using the SYBR Green PCR Master Mix® (Applied Biosystems) on an ABI7900HT Real-Time PCR system (Applied Biosystems). Oligonucleotides primer sequences are described in table 1. Based on data from the literature on bovine thyroid gland indicating that TATA-Box-binding protein (*TBP*) is one of the most stably expressed housekeeping genes in this tissue (Lisowski *et al.*, 2008), all qPCR data were normalized by TBP mRNA levels before being analysed with DART-PCR (Peirson *et al.*, 2003) .

2.9. Statistical analysis

The effect of the treatment on the time course of body weight was analysed using a two way ANOVA with the time of measure, the treatment and their interaction as fixed effect factor and the animal nested in the treatment as random effect factor.

For endocrine parameters and qPCR mRNA expression, variance homogeneity was tested and the effect of the treatment was analysed using an unpaired t-test using Systat 12® software for homogenous or non-homogenous variance. For TT4, as all available data consistently showed decreased total T4 in response to fipronil, a unilateral test was used to analyze this hormone.

3. Results

3.1. Preliminary experiment: fipronil toxicokinetic under repeated oral administrations

The time course of mean (± SD) plasma fipronil and fipronil sulfone concentrations following daily fipronil administrations for 14 days is shown in Figure 1. Twenty-four hours after the first and the third fipronil administrations, plasma fipronil concentrations had decreased to 68 ng/mL and 71 ng/mL, respectively but were still detectable. In contrast, 24 h after the tenth and the last administrations, plasma fipronil concentrations were below the limit of quantification of 5 ng/mL, indicating a time-dependent disposition of fipronil. Fipronil sulfone was detectable as early as 2 h after the first fipronil administration. Plasma fipronil sulfone concentrations, assayed 24 h after a fipronil administration and just before the next administration, progressively increased so that a steady-state seemed to be achieved around the third day of treatment. It is noteworthy that after the last administration, plasma fipronil sulfone concentrations decreased at a much slower rate than fipronil and was still detectable 100 h after the administration while fipronil was no longer detected at 8 h.

Modelling the time course of the plasma fipronil concentrations (representative example: Figure 1 bottom graph) showed that they fluctuated between two administrations and that the amplitude of these fluctuations decreased progressively between the first and the last administrations. Table 2 shows the mean (± SD) fipronil pharmacokinetic parameters 24 h after the first and the last administrations. Repeated administrations of fipronil increased the rate of fipronil elimination. Indeed, for the last (14^{th}) fipronil administration, the Cmax was 7.6-fold lower, the apparent clearance was 10-fold higher and the estimated half-life 10-fold shorter than after the first fipronil administration.

3.2. Main experiment: effects of fipronil treatment on endocrine parameters.

Fipronil treatment had no impact on the time course of body weights (data not shown). It did not have a significant effect on free T4 and total T3 mean concentrations but it significantly decreased total T4 and free T3 concentrations ($p \leq 0.05$; Fig. 2). A trend toward an increase in TSH concentration was also observed (23% increase, p=0.07).

3.3. Main experiment: fipronil and fipronil sulfone internal exposure

In agreement with toxicokinetic results from the preliminary experiment, 25 h after the last administration, we found almost exclusively fipronil sulfone in the plasma of exposed animals with concentrations as high as 3.1 ± 1 µg/ml while fipronil plasma concentrations remained close (5.9 ± 1.0 ng/ml, n=7) or below (3 animals out of 10) the assay LOQ of 2.5 ng/mL.

3.4. Main experiment: effects of fipronil treatment on thyroid transcriptome

We studied gene expression in the thyroid gland of 10 control and 10 fipronil exposed-rats using Agilent whole rat genome microarrays. By contrast with what was observed in the liver, fipronil had a relatively limited impact on the transcriptome of the thyroid gland in our conditions. In order to rule out technical issues with the microarray analysis, we also verified that consistent fold-changes (Fig 3A) and low signal variability (Fig 3B) were observed for Agilent spiked-in control RNAs. Given the fact that RNA expression / cDNA data from thyroid gland cells are scarce in databases and that it is likely that the first generation of Agilent "whole rat genome" microarrays used in this study only represents a fraction of the transcripts that are expressed in this highly specialized tissue, we accepted a higher chance of false positives (larger q-value=0.39). Two hundred and twenty eight probes showed p-value <0.005 (table 3). One hundred and seven were downregulated, while 121 were upregulated upon fipronil exposure. Finally, 157 differentially expressed probes corresponded to annotated genes and are presented in Table 3.

We did not observe any strong bias toward upregulation vs downregulation (Fig 3A). A heatmap of the individual z-scores for these transcripts in our 10 biological samples per group (Figure 3D) illustrates some variability in the response to fipronil exposure. Hierarchical clustering (Fig. 3D) of the

293 samples using the expression of the 228 selected probes showed a clear separation of the control and
294 fipronil-exposed thyroid samples. Only one sample exposed to fipronil was clustered in the wrong
295 group (with the controls) and displayed a limited transcriptomic response to the treatment (Fig 3D).
296 We analysed the enrichment of Gene Ontology biological processes among the genes that were either
297 down- or up-regulated upon fipronil exposure (Table 4 and 5). Upregulated genes were notably
298 involved in blood vessel and tissue dynamics, in detoxification processes and in signalling pathways
299 involved in response to xenobiotics and hormones (Table 5). Downregulated genes were mostly
300 enriched for functions associated with inflammatory and immune responses (Table 4). The Cy5 signal
301 associated with the microarray probe targeting the thyroglobulin gene (Tg) was saturated on all
302 microarrays and thus could not be analysed. The thyroid peroxidase (*Tpo*) is another key gene
303 involved in TH biosynthesis. The corresponding signal on the microarray showed some evidence of
304 upregulation (~30% increased signal, p=0.03) but did not reach our threshold for significance. Given
305 their key roles in TH biosynthesis, both genes were further analysed by qPCR. In addition, we studied
306 by qPCR other genes, present or not on the microarray, potentially involved in TH metabolism and/or
307 identified as fipronil targets in rodent liver (Roques *et al.*, 2013) including TH cell transporters.
308 Indeed, MCT8, a TH specific cell transporter is expressed in the thyroid gland where it could play a
309 substantial role in TH secretion (Di Cosmo *et al.*, 2010; Badziong *et al.*, 2017). Thus, we hypothezized
310 that other TH transporters, although less specific of TH, might as well be involved. This was an
311 attempt to provide novel insights not only on TH trafficking in and out the thyroid gland but also on
312 potential intrathyroidal targets of fipronil.
313
314 Figures 4 and 5 illustrate the qPCR results for all the tested genes involved in thyroid hormone
315 transportation/metabolism and cell/tissue dynamics, respectively. The expression of *Tg* appeared to be
316 significantly higher in fipronil-treated animals (p=0.04) and a trend for higher expression of the gene
317 encoding the NaI symporter (*Slc5a5*, p=0.063) was observed. *Tpo* expression did not appear to be
318 significantly modified in response to fipronil treatment (p=0.261). Among the genes encoding carriers
319 potentially involved in TH cellular transportation, the *Slco1a4* gene was occasionally expressed in 7
320 vehicle and 5 fipronil-treated animals. *Slco4a1* and *1a5* genes were expressed in all samples although

at low levels. None of the *Slco* genes that we investigated and that were expressed in the thyroid gland showed an effect of the fipronil treatment. *Sult1a1* and *1c3* were both expressed in all the samples. The level of expression of *Sult1a1* was much higher and only *Sult1a1* showed a differential expression with a 50% decrease in fipronil-treated animals (p=0.03). As for the genes potentially involved in thyroid cell dynamic and/or tumorigenesis, *Areg* (p=0.039) and *Ptgs1* (p=0.009) were upregulated in fipronil-treated animals (fig.5). A trend toward increased expression of *RhoA* gene in fipronil-treated animals was also observed (p=0.06).

4. **Discussion.**

Chemicals targeting the thyroid function are numerous and can act through many mechanisms (Duntas and Stathatos, 2015) including extrathyroidal sites of actions such as increased hepatic catabolism of TH, or intrathyroidal sites such as TPO inihibition like for example with PTU or, NIS inhibition like for example with perchlorates. There are more and more emerging evidences that, in human, exposure to environmental contaminants and potential endocrine disruptors such as polychlorinated biphenyls, organochlorine pesticides, diethylhexylphathale, bisphenol AF might contribute to the increased incidence of thyroid cancers (Lerro *et al.*, 2018);. In rat, it is assumed that the development of thyroid tumors in relation to thyroid disruption is the direct consequence of the compensatory reaction on the thyroid gland to counteract decreased concentrations of TH whatever the cause of this decrease. Therefore, understanding the sequence of events underlying those compensatory mechanisms, and more particularly increased cell dynamic, upon chemical interference is mandatory. The fact that current knowledge of thyroid cancer etiology is poor in human (Marotta *et al.*, 2016) further emphasizes this urgent need for a more comprehensive understanding of the interface between thyroid gland compensatory capacities and the risk of thyroid cancer.

In our previous studies, we showed that despite a very high increase in T4 clearance , the decrease in circulating thyroid hormones resulting from exposure to fipronil or its main metabolite fipronil sulfone remains limited (Leghait *et al.*, 2009; Roques *et al.*, 2012). This suggests that the thyroid gland might develop compensatory mechanisms leading to an increased synthesis and liberation of TH that could

contribute to overcome, at least in part, the dramatic increase in TH hepatic clearance. Our current results support this hypothesis by showing that thyroglobulin gene expression is increased. In addition a strong trend toward an increase expression of the *Slc5a5* gene (25%, p=0.06) was also observed. An upregulation of several genes with documented functions in cell/tissue dynamics was also evidenced that could be related to the hyperplasia described in toxicological non peer-reviewed survey of fipronil and one experimental study. In particular, we confirmed by RT-qPCR the upregulations upon fipronil exposure of *Areg* and *Ptgs1* by 3.4- and 1.8- fold, respectively.

Concerning fipronil exposure, our data are in agreement with data obtained by us and others in the rat and clearly show that repeated administrations of fipronil lead to an induction of its own metabolism/elimination resulting in a very limited exposure of the animals to fipronil itself toward the end of the experiment contrasting with an important exposure to fipronil main metabolite, fipronil sulfone (Leghait *et al.*, 2009; Roques *et al.*, 2012; Moser *et al.*, 2015). Fipronil sulfone *in vivo* is as active as fipronil at increasing TH hepatic catabolism resulting in an increased clearance of TH (Roques *et al.*, 2012). In addition, this metabolite is much more persistent and accumulates in the body in several species including human (Leghait *et al.*, 2009; Leghait *et al.*, 2010; Herin *et al.*, 2011; Cravedi *et al.*, 2013). Therefore, the sulfone metabolic pathway appears more as an aggravating factor than a detoxification mechanism. Fipronil sulfone should thus be taken into account when assessing the risk of fipronil for human health. Importantly, fipronil sulfone concentrations observed in our rodent studies were much higher than any reported concentration in human and thus the minimal toxic concentration remains to be determined. Whether fipronil by itself could induce the same effect on hepatic TH catabolism is a question that remains to be elucidated and that could be of some interest for species such as the cat with very limited sulfonation of fipronil (unpublished data). Nevertheless, in human as in rat, fipronil sulfone remains the main metabolite of fipronil formed *in vivo*.

The enriched GO functions for downregulated genes in the thyroid gland upon fipronil exposure are mostly related to inflammatory and immune responses (Table 4). One study showed that different types of fipronil exposure in rats (low vs high dose, acute vs repeated) resulted in contrasted effects on serum biomarkers for inflammatory and immune responses (Moser *et al.*, 2015). In this

study, a 14 day oral exposure to 5 mg/kg/day resulted in a moderate increase of serum chemokines *Cxcl10* and Ccl11 and of C-reactive protein (Crp), suggesting a systemic pro-inflammatory profile. The modest transcriptional response relative to the immune function that we observed in the thyroid is unlikely to be associated with alteration of the systemic inflammatory status. Rather, the downregulation of genes involved in inflammation and immunity that we observed within the thyroid gland itself likely represents a local response of the thyroid gland either directly to fipronil or to its potential systemic effects on immunity.

Genes upregulated in response to fipronil exposure were enriched for functions related to blood vessels and tissue dynamics (Table 5) which may be related to the thyroid hyperplasia (Wollman *et al.*, 1978) that has been evidenced in other studies. Enrichment was also noted for functions related to endocrine functions including the thyroid function.

We first focused our targeted RT-qPCR validations on genes that are limiting factors of TH biosynthesis: thyroperoxidase (*Tpo*), sodium-iodide symporter (*Slc5a5*) and thyroglobulin (*Tg*). The trend toward increased expression of *Slc5a5* in fipronil-treated animals is fully consistent with results from the regulatory toxicological evaluation of fipronil in the rat showing an increased uptake of iodine in treated animals. Fipronil and its persisting metabolite fipronil sulfone clearly increase the hepatic clearance of thyroid hormones in the rat. The resulting decrease in circulating TH reduces the negative feedback at the hypothalamo-pituitary level, thus increasing TSH secretion. A stimulation by TSH of the expression of genes involved in iodine supply to the thyreocytes and in the synthesis of TH precursor is currently considered as a key mechanism compensating xenobiotic-induced loss of TH. In the current study, the increase in TSH tended to be significant and was quite moderate while it was systematically reported in other studies. It is noteworthy that in the study of Roques et al., in which 20 animals were the same as in the current study, TSH concentration was significantly increased in fipronil-treated animals. Although likely, the hypothesis that increased TSH mediates at least in part the increased expression of key genes of TH biosynthesis still deserves more investigations. Interestingly, Phospholipase-C (*Plc*) was also among the upregulated genes upon fipronil exposure. TSH receptor-Gq protein phospholipase C cascade activation by TSH is one signalling pathway for TSH effect on thyroid follicular cell in human (Laurent *et al.*, 1987) and rat FRTL-5 cells (Kim *et al.*,

2002). As fipronil and/or its metabolite can accumulate in the thyroid with concentrations about 3-fold higher than the blood concentrations 72 h after a single administration (Cravedi *et al.*, 2013), a direct action of the compounds remains possible. There are some evidence that expression of *Tpo*, *Slc5a5* and *Tg* can be modulated independently of TSH mediation by direct exposure to pollutants such as TCDD and or dioxin-like coplanar PCBs in chicken thyroid explants (Katarzynska *et al.*, 2015). Furthermore, this study showed that not all 3 genes were modulated in the same manner by these pollutants. For example, TCDD did not modify *Slc5a5* expression but increased *Tpo* and *Tg* gene expressions while PCB126 decreased *Slc5a5* expression while increasing *Tpo* and *Tg* expressions. This could be consistent with the fact that in our study only *Tg* gene and in a lesser extent *Slc5a5* gene expressions were upregulated although *Tpo* is also known as a privileged target of TSH.

Data regarding intrathyroidal transportation and metabolism (other than synthesis) of TH are scarce although it can reasonably be assumed that intrathyroidal metabolism of TH might also be a regulatory process for controlling TH systemic concentrations. Our data indicate that *Sult 1A1* and *1A3* expressions are significant in the rat thyroid. This is consistent with data in human thyroid showing that SULTs expression is overall very low or undetectable except for SULTs 1A1, 1A3, 1B1 and 1C2 (Nishimura and Naito, 2006). SULT 1A*1* mRNA expression was decreased in fipronil-treated animals. In hyperthyroid disorders in humans, it was proposed that increased TH levels induces intrathyroidal SULT activity which in turn could act as a protective mechanism enhancing deiodination /inactivation of excess hormone (Ebmeier and Anderson, 2004). It could thus be hypothesized that the decrease in thyroid SULT1A1 mRNA expression, if associated to a decrease in protein content and activity, in the thyroid may decrease intrathyroidal metabolization of produced TH and could represent another compensatory mechanism allowing to partially overcome the consequences of accelerated TH elimination.

We also identified genes encoding factors involved in cell/tissue dynamics that are upregulated in the thyroid of fipronil-treated rats. In particular, we validated by qPCR the upregulation of 3 factors related to dysregulation of the thyroid gland cellular dynamic: *Areg*, a growth factor often

described in different types of cancer, prostaglandin synthase1 (*Ptgs1*) a key enzyme in prostaglandin synthesis and, a trend toward increased expression of the GTPase *RhoA*.

AREG is a growth factor that belongs to the EGF-related peptides that binds and activates the ERB-1 receptor. These factors are often involved in promoting the proliferation of tumoral cells. Coexpression of ERB-1 and some of its binding proteins has been hypothesized as a putative autocrine pathway of cell proliferation in papillary thyroid carcinoma in human. Although there is no evidence yet that such mechanisms could mediate follicular hyperplasia and/or subsequent follicular tumours in rodents, this hypothesis deserves further attention.

Prostaglandins are key actors of inflammatory responses in several organs, including the thyroid gland in which they can promote cell proliferation. The rate limiting step of their synthesis is catalysed by prostaglandin synthases (PTGS) 1 and 2. In FRTL-5 rat thyroid cells, TSH regulates all three steps of prostaglandin synthesis including PTGS mRNA expression and activity. Interestingly, PTGS enzymes have been implicated in different cell culture models in the mediation of some effects of TSH and/or TSH-like immunoglobulins found in autoimmune diseases. PTGS1 is thought to sustain the production of PGE2 in primary human thyroid endothelial cells and seems to play a key role in cell proliferation and tumorigenesis in nude mice grafted with human medullary thyroid cancer cells. It is noteworthy that other genes related to prostaglandin metabolism appeared to be upregulated in the thyroid gland of fipronil-treated animals (Table 3: Prostaglandin reductase 1: *Ptgr1*; Phospholipase C: *Plcb*, *Abcc4* encoding the MRP4 efflux transporter of PGE2, *Slc51a* encoding a cell transporter of PGE2). Whether the increased *Ptgs1* expression observed in our study contributes to hyperplasia or even follicular carcinoma development that occurs later in fipronil-treated animals warrants further investigations.

In conclusion, our study shows that the increased hepatic clearance of thyroid hormones induced by fipronil and/or its major metabolite, fipronil sulfone, triggers compensatory pathways in the thyroid gland related to both TH synthesis and cell and tissue dynamics. The expression of most of the differentially regulated genes that we investigated might be modulated by TSH. However, because of the accumulation of fipronil residues in the thyroid and because the increase in TSH did not reach

significance in the current study, it is not possible to conclude on which of those regulation are TSH-mediated or not. Importantly, in Human, exposure to bisphenol AF and diethylhexylphthalate increases the susceptibility to develop differentiated thyroid cancer in patients with thyroid nodules (Marotta *et al.*, 2019), without correlation with higher TSH levels. This suggests that thyroid cancer development related to exposure to environmental contaminants might possibly proceed from direct actions in the thyroid gland rather than compensatory processes triggered by increased TSH. This hypothesis, raised in an already pathological situation, will require further investigation. One possibility to address this question could be to compare the effect of fipronil on the thyroid gland histology and transcriptomic profiles in rats treated with fipronil alone or with fipronil and low levels of T4 to restore the full negative feedback on TSH secretion.

Our results provide new insights on the intimate molecular mechanisms underlying thyroid hyperplasia and the subsequent tumoral processes observed in rats following long-term fipronil treatments. Further high-throughput studies on other molecules affecting thyroid function are required to understand which pathways represent common or specific targets of thyroid disruptors.

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607

608 ***Figure legends***

609 **Fig. 1:** Top graph: time course of fipronil and its main metabolite fipronil sulfone mean (±SD) plasma
610 concentrations in rats (n=3) treated for 14 days (arrows) with daily oral administration of fipronil (3
611 mg/kg/d). Bottom graph: predicted detailed fluctuations of fipronil plasma concentrations (line) in one
612 representative rat obtained by fitting the observed data (dot) with a monoexponential equation and
613 clearance defined as a time–dependent variable. Fipronil and/or fipronil sulfone were assayed in blood
614 samples collected serially after the first and the last administrations and once just before the forth and
615 the tenth ones.

616

617

618 **Fig. 2:** Mean (±SE) thyroid hormone and TSH concentration in adult female rats treated with vehicle
619 or fipronil (3 mg/kg/day, *po*) for 14 days. Hormones were assayed in blood collected approximately 24
620 h after the last fipronil administration. *: different from vehicle $p \leq 0.05$. For TT4, as all available data
621 consistently showed decreased totalT4 in response to fipronil, a unilateral test was used to analyze this
622 hormone.

623

624 **Fig.3:** Effect of fipronil on the rat thyroid transcriptome.
625 A. Using the same data analysis pipeline as for the rest of the genes, we analyzed the differential
626 expression of 10 spiked-in RNA provided by Agilent in defined ratios between 2 spike mixes. The plot

represents the observed vs expected log2 (fold changes) for the 10 spikes. Except for the spikes present at a 1:1 ratio, all changes were highly significant ($p<2e^{-8}$).

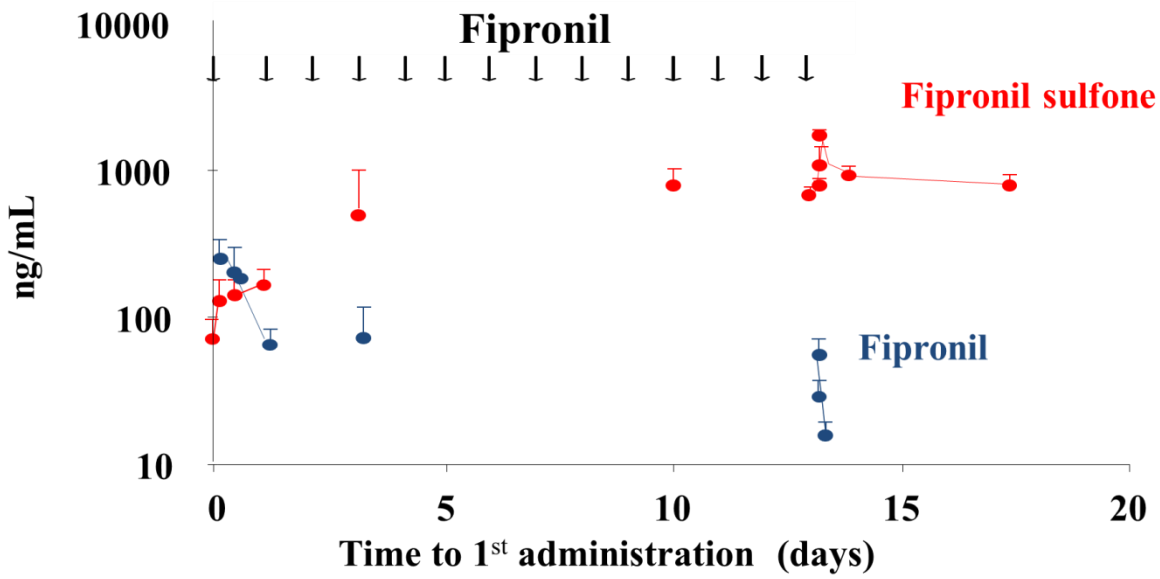
B. For the 10 spikes presented in panel A and the 2 different mixes (Cy3 and Cy5), we plotted the coefficient of variation vs the median log2 (normalized intensity) to illustrate the low signal variability across the range of spikes concentrations.

C. Volcano plot showing log2 fold-change (x-axis) vs significance (y-axis) for all pre-filtered genes on 4x44K Agilent microarray and highlighting the 223 selected genes with a significant gene expression change ($p<0.005$) in adult female rats exposed to fipronil (3 mg/Kg/d, 14 days).

D. Heatmap for all samples (n=10 per group) of the z-scores of the 223 significantly modulated genes in the thyroid gland of female rats exposed to fipronil. A summary of the main GO biological processes enriched among the up- or downregulated genes is indicated in the right of the heatmap (see tables 4 and 5 for details).

Fig.4: Relative mRNA expression (mean ± SE) of genes involved in thyroid hormone metabolism or transportation in vehicle or fipronil-treated female rats (3 mg/ kg/day po for 14 days). For some genes in some animals not enough RNA was left to run the qPCR due to the small size of the thyroid gland. *Slco1A4* was not expressed in all samples, so the graph depicts only the values of the detectable samples. N= number of samples assayed.

Fig. 5: Relative mRNA expression (mean ± SE) of genes possibly involved in cell dynamics or cancer within the thyroid in vehicle or fipronil-treated female rats (3 mg/ kg/d po for 14 days). The number of samples assayed for each gene is indicated (n).


10000
1000
100
10
ng/mL
Fipronil
Fipronil sulfone
Fipronil
0
5
10
15
20
Time to 1st administration (days)

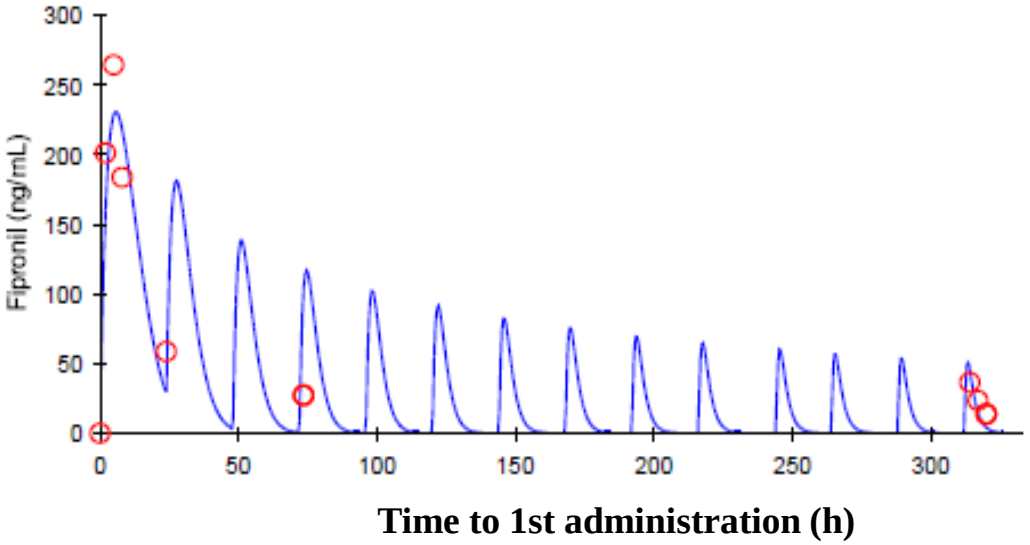

300
250
200
150
100
50
0
Fipronil (ng/mL)
0
50
100
150
200
250
300
Time to 1st administration (h)

Figure 1

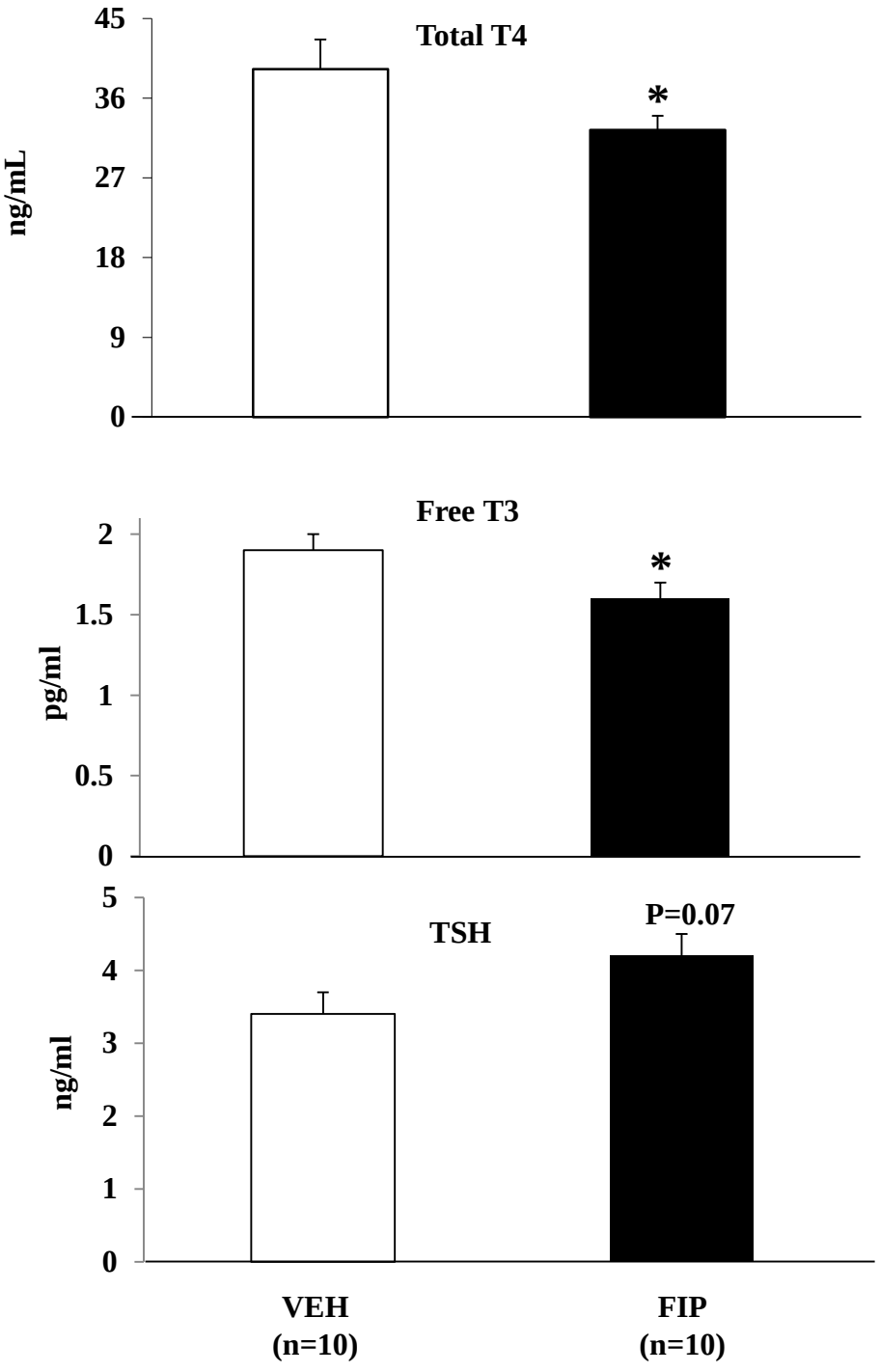

Total T4
45
36
27
18
9
0
ng/mL
*
Free T3
2
1.5
1
0.5
0
pg/ml
*
TSH
5
4
3
2
1
0
ng/ml
P=0.07
VEH
(n=10)
FIP
(n=10)

Figure 2

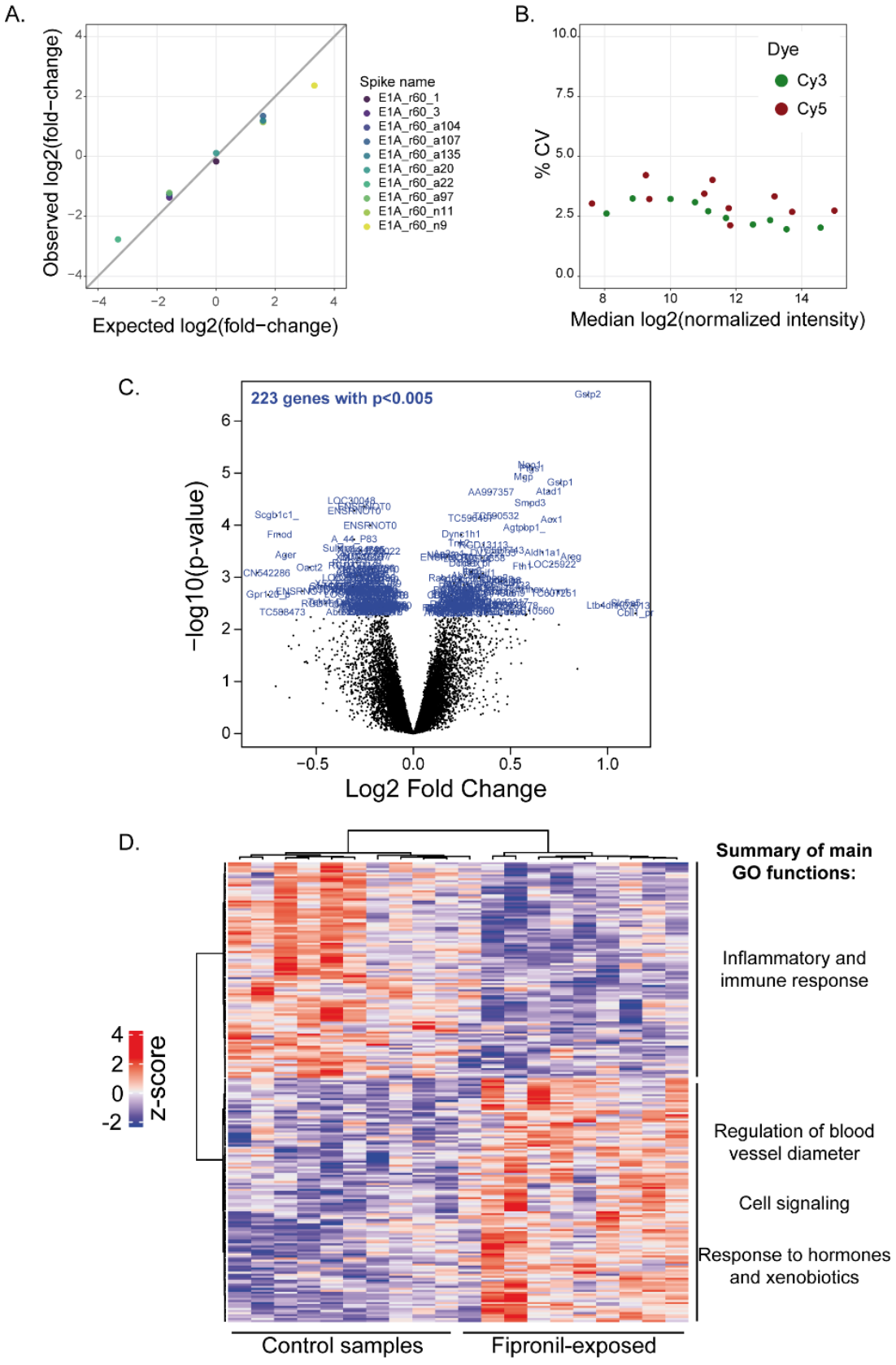

A.
Observed log2(fold−change)
Expected log2(fold−change)
Spike name
E1A_r60_1
E1A_r60_3
E1A_r60_a104
E1A_r60_a107
E1A_r60_a135
E1A_r60_a20
E1A_r60_a22
E1A_r60_a97
E1A_r60_n11
E1A_r60_n9
B.
% CV
Median log2(normalized intensity)
Dye
Cy3
Cy5
C.
223 genes with p<0.005
−log10(p-value)
Log2 Fold Change
Gstp2
D.
z-score
Control samples
Fipronil-exposed
Summary of main GO functions:
Inflammatory and immune response
Regulation of blood vessel diameter
Cell signaling
Response to hormones and xenobiotics

Figure 3

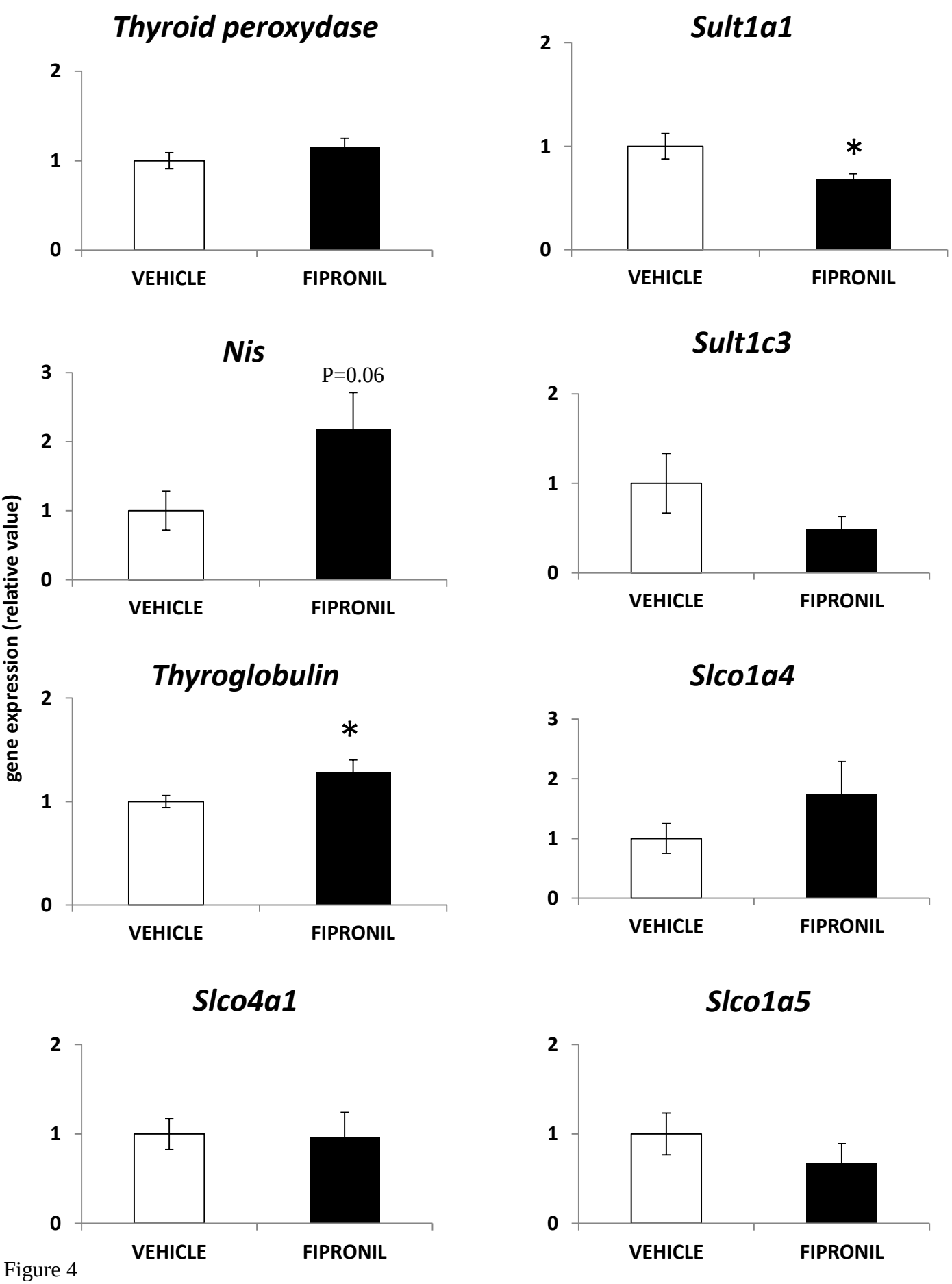

Thyroid peroxydase
Sult1a1
Nis
P=0.06
Sult1c3
Thyroglobulin
Slco1a4
Slco4a1
Slco1a5
gene expression (relative value)
VEHICLE
FIPRONIL
*

Figure 4

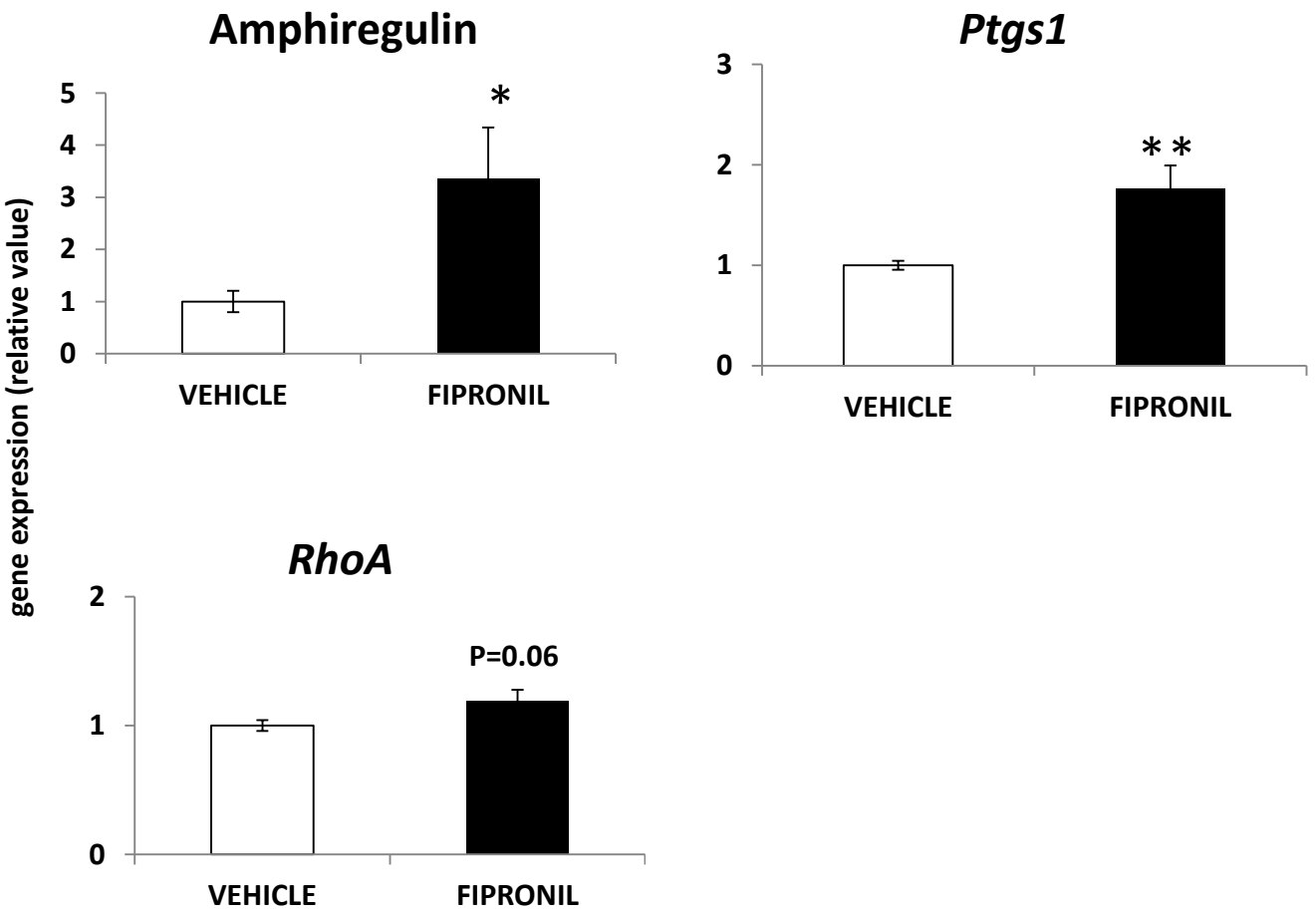

Amphiregulin
gene expression (relative value)
5
4
3
2
1
0
*
VEHICLE
FIPRONIL
Ptgs1
3
2
1
0
**
VEHICLE
FIPRONIL
RhoA
2
1
0
P=0.06
VEHICLE
FIPRONIL

Figure 5

Table 1. Oligonucleotide primer sequences for real time PCR.

Gene	NCBI RefSeq	Forward primer (5'-3')	Reverse primer (5'-3')
Adra1b	NM_016991	TCTTATGTTGGCTCCCCTTCT	ACGGGTAGATGATGGGATTG
Areg	NM_017123	CGGAGGAGTATGATAACGAACC	CCTTTGCCTCCCTTTTTCTT
Slc5a5	NM_052983	TCCACAGGAATCATCTGCAC	AAGCCAACGAGCATTACCAC
Ptgs1	NM_017043	TTGGCCTGAAGCCTTACACT	TGTCACCATATAGCTCCTCCAA
Rhoa	NM_057132	GAAGTCAAGCATTTCTGTCCAA	TGGCTAACTCCCGCCTTGTGT
Tbp	NM_001004198	ACTTCGTGCCAGAAATGCTGAA	GCAGTTGTTCGTGGCTCTCT
Tg	NM_030988	GCTTATCAACAGGGCAAAGG	TTCTGCAGTGCCTGGTAAAA
Tpo	NM_019353	AATTTTCCTCCCTTTCCTCCT	AGACTGCATTGTCCACCAGA
Sult1a1	NM_031834	TGAGCACCCGGAGGCA	TAGCGGTGGACGGGAGAA
Sult1c2a	NM_001013177	TCTGCTTCTGCCCTTGAGGTAT	ATGATGTCTCAGGGAAGAAGGTTT
Sult1c3	NM_031732	CCTTATTGCAACCTATGCAAAAGC	CATGTCCACAATTTCCTGCG
Slco1a1	NM_017111	AGATTAGACTTCTCACTCCTGTGTCATT	TAATAACCTGATTAAGTTTGTCAGTGCTC
Slco1a4	NM_131906	AACTTTCCTATTGCAGAAATATCATTTAGA	TGCAGAGGATAAATCAAAACAAACTAAC
Slco1a5	NM_030838	GGGATTTTAGAAACAGGAAAGGTCT	CCAGCGGGTATCAGTGGG
Slco4a1	NM_133608	TTTGGCAAGACTGTCAGAGACCT	CAGGCAGAGCAGGATGAATGT
Slco1b3	NM_001270587	CAAACAAGGTTCTGCGATGGAT	CTACATATGCAAAGCACTAGGTGGAG

Table 2: Mean (± standard deviation) fipronil pharmacokinetic parameters in rats (n = 3) following oral daily repeated administrations (3 mg/kg/day for 14 days).

	1st fipronil administration	14th fipronil administration
Cmax (ng/mL)	599 ± 145	79 ± 42
$t_{1/2}$ (h)	4.02 ± 1.88	0.38 ± 0.18
Apparent clearance (mL/min/kg)	11.80 ± 6.17	127.93 ± 71.89

Fipronil pharmacokinetic parameters were estimated with a monoexponential model for repeated administrations with a first-order absorption phase and a time-dependent apparent clearance.

Table 3: list of annotated genes differentially regulated in fipronil-treated adult female rats

ProbeName	Gene Symbol	GeneName	log2FC	p-value	q-value	Description [target ID]
A_44_P290591	Nectin2	nectin cell adhesion molecule 2	0.14	0.0039	0.3633	Rattus norvegicus nectin cell adhesion molecule 2 (Nectin2), mRNA [NM_001012064]
A_44_P192548	Gtpbp2	GTP binding protein 2	0.15	0.0042	0.3719	Rattus norvegicus GTP binding protein 2 (Gtpbp2), mRNA [NM_001013225]
A_42_P623151	Dync1li2	dynein, cytoplasmic 1 light intermediate chain 2	0.16	0.0032	0.3520	Rattus norvegicus dynein, cytoplasmic 1 light intermediate chain 2 (Dync1li2), mRNA [NM_031026]
A_44_P380384	Ripply3	ripply transcriptional repressor 3	0.16	0.0040	0.3657	Rattus norvegicus ripply transcriptional repressor 3 (Ripply3), mRNA [NM_001105892]
A_44_P554795	Rab11a	RAB11a, member RAS oncogene family	0.17	0.0010	0.2839	Rattus norvegicus RAB11a, member RAS oncogene family (Rab11a), mRNA [NM_031152]
A_44_P198620	Nos3	nitric oxide synthase 3	0.17	0.0033	0.3520	Rattus norvegicus nitric oxide synthase 3 (Nos3), mRNA [NM_021838]
A_44_P236566	Neurl2	neuralized E3 ubiquitin protein ligase 2	0.17	0.0004	0.2006	Rattus norvegicus neuralized E3 ubiquitin protein ligase 2 (Neurl2), mRNA [NM_001107802]
A_44_P104444 1	Phtf1	putative homeodomain transcription factor 1	0.17	0.0004	0.2006	Rattus norvegicus putative homeodomain transcription factor 1 (Phtf1), mRNA [NM_001191102]
A_44_P448002	Ccdc102a	coiled-coil domain containing 102A	0.17	0.0038	0.3610	Rattus norvegicus coiled-coil domain containing 102A (Ccdc102a), mRNA [NM_001108437]
A_42_P704370	Arl6ip5	ADP-ribosylation factor like GTPase 6 interacting protein 5	0.18	0.0049	0.3884	ADP-ribosylation factor like GTPase 6 interacting protein 5 [Source:RGD Symbol;Acc:708572] [ENSRNOT00000010185]
A_44_P136285	Jam3	junctional adhesion molecule 3	0.18	0.0049	0.3884	Rattus norvegicus junctional adhesion molecule 3 (Jam3), mRNA [NM_001004269]
A_43_P14179	Ap2m1	adaptor-related protein complex 2, mu 1 subunit	0.18	0.0004	0.2006	Rattus norvegicus adaptor-related protein complex 2, mu 1 subunit (Ap2m1), mRNA [NM_053837]
A_43_P13419	Ppp1r14b	protein phosphatase 1, regulatory (inhibitor) subunit 14B	0.18	0.0044	0.3821	Rattus norvegicus protein phosphatase 1, regulatory (inhibitor) subunit 14B (Ppp1r14b), mRNA [NM_172045]
A_44_P550581	Dmpk	DM1 protein kinase	0.18	0.0041	0.3678	dystrophia myotonica-protein kinase [Source:RGD Symbol;Acc:1309825] [ENSRNOT00000020428]

ProbeName	Gene Symbol	GeneName	log2FC	p-value	q-value	Description [target ID]
A_44_P358203	Mark1	microtubule affinity regulating kinase 1	0.20	0.0021	0.3025	Rattus norvegicus microtubule affinity regulating kinase 1 (Mark1), mRNA [NM_053947]
A_44_P1054280	Pck2	phosphoenolpyruvate carboxykinase 2 (mitochondrial)	0.20	0.0022	0.3025	Rattus norvegicus phosphoenolpyruvate carboxykinase 2 (mitochondrial) (Pck2), mRNA [NM_001108377]
A_43_P22174	Wdr1	WD repeat domain 1	0.20	0.0029	0.3520	Rattus norvegicus WD repeat domain 1 (Wdr1), mRNA [NM_001014135]
A_44_P160888	Ppp1r14b	protein phosphatase 1, regulatory (inhibitor) subunit 14B	0.20	0.0039	0.3633	Rattus norvegicus protein phosphatase 1, regulatory (inhibitor) subunit 14B (Ppp1r14b), mRNA [NM_172045]
A_44_P401030	Clec14a	C-type lectin domain containing 14A	0.21	0.0033	0.3520	Rattus norvegicus C-type lectin domain family 14, member A (Clec14a), mRNA [NM_001014077]
A_44_P1003212	Igf2r	insulin-like growth factor 2 receptor	0.21	0.0013	0.2996	Rattus norvegicus insulin-like growth factor 2 receptor (Igf2r), mRNA [NM_012756]
A_44_P546537	Nucb2	nucleobindin 2	0.21	0.0027	0.3464	Rattus norvegicus nucleobindin 2 (Nucb2), mRNA [NM_021663]
A_44_P311455	Nrg2	neuregulin 2	0.21	0.0035	0.3520	Rattus norvegicus neuregulin 2 (Nrg2), mRNA [NM_001136151]
A_44_P423803	Actb	actin, beta	0.21	0.0040	0.3642	Rattus norvegicus actin, beta (Actb), mRNA [NM_031144]
A_44_P429455	Lama5	laminin subunit alpha 5	0.22	0.0031	0.3520	Rattus norvegicus laminin subunit alpha 5 (Lama5), mRNA [NM_001191609]
A_44_P1039678	Setd4	SET domain containing 4	0.22	0.0015	0.2996	Rattus norvegicus SET domain containing 4 (Setd4), mRNA [NM_001113747]
A_42_P772965	Aldh7a1	aldehyde dehydrogenase 7 family, member A1	0.22	0.0049	0.3884	Rattus norvegicus aldehyde dehydrogenase 7 family, member A1 (Aldh7a1), mRNA [NM_001271105]
A_44_P945336	Phf20l1	PHD finger protein 20-like 1	0.22	0.0022	0.3025	Rattus norvegicus PHD finger protein 20-like 1 (Phf20l1), mRNA [NM_001271439]
A_44_P1026651	Itm2b	integral membrane protein 2B	0.23	0.0027	0.3464	Rattus norvegicus integral membrane protein 2B (Itm2b), mRNA [NM_001006963]
A_43_P14782	Tnk2	tyrosine kinase, non-receptor, 2	0.23	0.0002	0.1870	Rattus norvegicus tyrosine kinase, non-receptor, 2 (Tnk2), mRNA [NM_001008336]
A_44_P303155	Agtpbp1	ATP/GTP binding protein 1	0.24	0.0011	0.2895	Rattus norvegicus ATP/GTP binding protein 1 (Agtpbp1), mRNA [NM_001106100]

ProbeName	Gene Symbol	GeneName	log2FC	p-value	q-value	Description [target ID]
A_44_P179787	Panx2	pannexin 2	0.24	0.0033	0.3520	Rattus norvegicus pannexin 2 (Panx2), mRNA [NM_199409]
A_44_P226601	F8	coagulation factor VIII	0.24	0.0046	0.3841	Rattus norvegicus coagulation factor VIII (F8), mRNA [NM_183331]
A_44_P224547	Plcb1	phospholipase C beta 1	0.25	0.0041	0.3678	Rattus norvegicus phospholipase C beta 1 (Plcb1), mRNA [NM_001077641]
A_44_P348868	Dync1h1	dynein cytoplasmic 1 heavy chain 1	0.25	0.0002	0.1450	Rattus norvegicus dynein cytoplasmic 1 heavy chain 1 (Dync1h1), mRNA [NM_019226]
A_44_P110110	Cst3	cystatin C	0.25	0.0029	0.3520	Rattus norvegicus cystatin C (Cst3), mRNA [NM_012837]
A_44_P618632	Stx2	syntaxin 2	0.25	0.0032	0.3520	syntaxin 2 [Source:RGD Symbol;Acc:2558] [ENSRNOT00000001242]
A_42_P609263	Lrrc42	leucine rich repeat containing 42	0.25	0.0029	0.3520	Rattus norvegicus leucine rich repeat containing 42 (Lrrc42), mRNA [NM_001025653]
A_44_P1033459	Uxs1	UDP-glucuronate decarboxylase 1	0.25	0.0020	0.3025	Rattus norvegicus UDP-glucuronate decarboxylase 1 (Uxs1), mRNA [NM_139336]
A_44_P149190	Ftl1	ferritin light chain 1	0.26	0.0019	0.3025	Rattus norvegicus ferritin light chain 1 (Ftl1), mRNA [NM_022500]
A_44_P483360	Nrip3	nuclear receptor interacting protein 3	0.26	0.0021	0.3025	Rattus norvegicus nuclear receptor interacting protein 3 (Nrip3), mRNA [NM_001108498]
A_42_P589025	Slc12a5	solute carrier family 12 member 5	0.26	0.0032	0.3520	Rattus norvegicus solute carrier family 12 member 5 (Slc12a5), mRNA [NM_134363]
A_42_P777285	Rell1	RELT-like 1	0.26	0.0014	0.2996	RELT-like 1 [Source:RGD Symbol;Acc:1307468] [ENSRNOT00000076611]
A_44_P592248	Slc8b1	solute carrier family 8 member B1	0.26	0.0024	0.3171	Rattus norvegicus solute carrier family 8 member B1 (Slc8b1), mRNA [NM_001017488]
A_43_P12841	Enpp1	ectonucleotide pyrophosphatase/phosphodiesterase 1	0.26	0.0017	0.2996	Rattus norvegicus ectonucleotide pyrophosphatase/phosphodiesterase 1 (Enpp1), mRNA [NM_053535]
A_44_P1007105	Plpp3	phospholipid phosphatase 3	0.26	0.0035	0.3520	Rattus norvegicus phospholipid phosphatase 3 (Plpp3), mRNA [NM_138905]
A_42_P541034	Ftl1	ferritin light chain 1	0.26	0.0021	0.3025	Rattus norvegicus ferritin light chain 1 (Ftl1), mRNA [NM_022500]
A_44_P257526	Abcc4	ATP binding cassette subfamily C member 4	0.27	0.0009	0.2839	Rattus norvegicus ATP binding cassette subfamily C member 4 (Abcc4), mRNA [NM_133411]
A_42_P805179	Gfpt1	glutamine fructose-6-phosphate transaminase 1	0.27	0.0039	0.3633	glutamine fructose-6-phosphate transaminase 1 [Source:RGD Symbol;Acc:1549703] [ENSRNOT00000090827]

ProbeName	Gene Symbol	GeneName	log2FC	p-value	q-value	Description [target ID]
A_44_P458570	Fut4	fucosyltransferase 4	0.27	0.0017	0.2996	Rattus norvegicus fucosyltransferase 4 (Fut4), mRNA [NM_022219]
A_44_P1031571	Map1b	microtubule-associated protein 1B	0.27	0.0028	0.3501	Rattus norvegicus microtubule-associated protein 1B (Map1b), mRNA [NM_019217]
A_44_P166206	Nostrin	nitric oxide synthase trafficking	0.27	0.0036	0.3570	Rattus norvegicus nitric oxide synthase trafficking (Nostrin), mRNA [NM_001024260]
A_42_P542744	Dchs1	dachsous cadherin-related 1	0.29	0.0006	0.2332	Rattus norvegicus dachsous cadherin-related 1 (Dchs1), mRNA [NM_001107544]
A_42_P762814	Dynlt1	dynein light chain Tctex-type 1	0.30	0.0005	0.2330	Rattus norvegicus dynein light chain Tctex-type 1 (Dynlt1), mRNA [NM_031318]
A_44_P699895	Mark1	microtubule affinity regulating kinase 1	0.30	0.0001	0.0924	microtubule affinity regulating kinase 1 [Source:RGD Symbol;Acc:619882] [ENSRNOT00000080309]
A_44_P149188	Ftl1	ferritin light chain 1	0.30	0.0008	0.2682	Rattus norvegicus ferritin light chain 1 (Ftl1), mRNA [NM_022500]
A_44_P306639	Dap	death-associated protein	0.30	0.0025	0.3292	Rattus norvegicus death-associated protein (Dap), mRNA [NM_022526]
A_44_P288185	Itpr3	inositol 1,4,5-trisphosphate receptor, type 3	0.30	0.0008	0.2668	Rattus norvegicus inositol 1,4,5-trisphosphate receptor, type 3 (Itpr3), mRNA [NM_013138]
A_43_P17148	Slc51a	solute carrier family 51, alpha subunit	0.31	0.0032	0.3520	Rattus norvegicus solute carrier family 51, alpha subunit (Slc51a), mRNA [NM_001107087]
A_44_P591224	Spg7	SPG7, paraplegin matrix AAA peptidase subunit	0.33	0.0046	0.3841	SPG7, paraplegin matrix AAA peptidase subunit [Source:RGD Symbol;Acc:727940] [ENSRNOT00000091712]
A_44_P240274	Emcn	endomucin	0.33	0.0028	0.3502	Rattus norvegicus endomucin (Emcn), mRNA [NM_001004228]
A_42_P689013	Txn1	thioredoxin 1	0.34	0.0010	0.2839	Rattus norvegicus thioredoxin 1 (Txn1), mRNA [NM_053800]
A_44_P412236	Stag3	stromal antigen 3	0.34	0.0024	0.3164	Rattus norvegicus stromal antigen 3 (Stag3), mRNA [NM_053730]
A_43_P17743	Pir	pirin	0.34	0.0010	0.2839	Rattus norvegicus pirin (Pir), mRNA [NM_001009474]
A_42_P799113	Egfl7	EGF-like-domain, multiple 7	0.34	0.0047	0.3841	Rattus norvegicus EGF-like-domain, multiple 7 (Egfl7), mRNA [NM_139104]
A_43_P16753	Man2a1	mannosidase, alpha, class 2A, member 1	0.35	0.0016	0.2996	Rattus norvegicus mannosidase, alpha, class 2A, member 1 (Man2a1), mRNA [NM_012979]

ProbeName	Gene Symbol	GeneName	log2FC	p-value	q-value	Description [target ID]
A_44_P769800	Flt1	FMS-related tyrosine kinase 1	0.35	0.0004	0.2006	Rattus norvegicus FMS-related tyrosine kinase 1 (Flt1), transcript variant 2, mRNA [NM_001309381]
A_44_P205572	Atpif1	ATPase inhibitory factor 1	0.36	0.0009	0.2744	Rattus norvegicus ATPase inhibitory factor 1 (Atpif1), mRNA [NM_012915]
A_44_P1048380	Fam46a	family with sequence similarity 46, member A	0.36	0.0002	0.1948	Rattus norvegicus family with sequence similarity 46, member A (Fam46a), mRNA [NM_001106844]
A_43_P12029	Rgs5	regulator of G-protein signaling 5	0.38	0.0037	0.3595	Rattus norvegicus regulator of G-protein signaling 5 (Rgs5), mRNA [NM_019341]
A_43_P15662	Tfrc	transferrin receptor	0.38	0.0047	0.3841	Rattus norvegicus transferrin receptor (Tfrc), mRNA [NM_022712]
A_44_P1057585	Htatip2	HIV-1 Tat interactive protein 2	0.38	0.0035	0.3520	Rattus norvegicus HIV-1 Tat interactive protein 2 (Htatip2), mRNA [NM_001106263]
A_44_P346491	Srsf5	serine and arginine rich splicing factor 5	0.38	0.0045	0.3841	Rattus norvegicus serine and arginine rich splicing factor 5 (Srsf5), transcript variant 2, mRNA [NM_019257]
A_44_P325133	Adamtsl2	ADAMTS-like 2	0.39	0.0035	0.3520	ADAMTS-like 2 [Source:RGD Symbol;Acc:1305459] [ENSRNOT00000036995]
A_44_P925373	Adra2a	adrenoceptor alpha 2A	0.40	0.0000	0.0593	Rattus norvegicus adrenoceptor alpha 2A (Adra2a), mRNA [NM_012739]
A_44_P578390	Abcc4	ATP binding cassette subfamily C member 4	0.41	0.0003	0.2006	PREDICTED: Rattus norvegicus ATP binding cassette subfamily C member 4 (Abcc4), transcript variant X1, mRNA [XM_008770939]
A_44_P840118	LOC102555426	uncharacterized LOC102555426	0.41	0.0020	0.3025	PREDICTED: Rattus norvegicus uncharacterized LOC102555426 (LOC102555426), ncRNA [XR_349314]
A_44_P116591	Cx3cl1	C-X3-C motif chemokine ligand 1	0.41	0.0016	0.2996	Rattus norvegicus C-X3-C motif chemokine ligand 1 (Cx3cl1), mRNA [NM_134455]
A_43_P12242	Plpp1	phospholipid phosphatase 1	0.43	0.0011	0.2845	Rattus norvegicus phospholipid phosphatase 1 (Plpp1), mRNA [NM_022538]
A_44_P992854	Rhoa	ras homolog family member A	0.43	0.0018	0.3025	ras homolog family member A [Source:RGD Symbol;Acc:619921] [ENSRNOT00000071664]
A_44_P867229	Btbd3	BTB domain containing 3	0.44	0.0012	0.2895	PREDICTED: Rattus norvegicus BTB domain containing 3 (Btbd3), transcript variant X1, mRNA [XM_006235099]
A_44_P668572	Slc35d1	solute carrier family 35 member D1	0.45	0.0003	0.2006	solute carrier family 35 member D1 [Source:RGD Symbol;Acc:1309843] [ENSRNOT00000032126]

ProbeName	Gene Symbol	GeneName	log2FC	p-value	q-value	Description [target ID]
A_44_P960765	Chodl	chondrolectin	0.45	0.0012	0.2895	Rattus norvegicus chondrolectin (Chodl), mRNA [NM_001105894]
A_42_P833096	Kcnj16	potassium voltage-gated channel subfamily J member 16	0.48	0.0012	0.2895	Rattus norvegicus potassium voltage-gated channel subfamily J member 16 (Kcnj16), mRNA [NM_053314]
A_42_P455531	Chrm3	cholinergic receptor, muscarinic 3	0.49	0.0045	0.3841	Rattus norvegicus cholinergic receptor, muscarinic 3 (Chrm3), mRNA [NM_012527]
A_42_P762829	Cebpd	CCAAT/enhancer binding protein delta	0.51	0.0048	0.3856	Rattus norvegicus CCAAT/enhancer binding protein delta (Cebpd), mRNA [NM_013154]
A_44_P419898	Cldn9	claudin 9	0.51	0.0020	0.3025	Rattus norvegicus claudin 9 (Cldn9), mRNA [NM_001011889]
A_44_P851230	Flt1	FMS-related tyrosine kinase 1	0.53	0.0035	0.3520	Rattus norvegicus FMS-related tyrosine kinase 1 (Flt1), transcript variant 1, mRNA [NM_019306]
A_44_P292634	Fth1	ferritin heavy chain 1	0.55	0.0016	0.2996	Rattus norvegicus ferritin heavy chain 1 (Fth1), mRNA [NM_012848]
A_44_P351546	Fth1	ferritin heavy chain 1	0.56	0.0006	0.2429	Rattus norvegicus ferritin heavy chain 1 (Fth1), mRNA [NM_012848]
A_42_P588944	Mgp	matrix Gla protein	0.57	0.0000	0.0550	Rattus norvegicus matrix Gla protein (Mgp), mRNA [NM_012862]
A_43_P16617	Agtpbp1	ATP/GTP binding protein 1	0.57	0.0001	0.1213	Rattus norvegicus ATP/GTP binding protein 1 (Agtpbp1), mRNA [NM_001106100]
A_42_P508085	Tmem204	transmembrane protein 204	0.60	0.0044	0.3821	Rattus norvegicus transmembrane protein 204 (Tmem204), mRNA [NM_001009620]
A_44_P476733	Nqo1	NAD(P)H quinone dehydrogenase 1	0.60	0.0000	0.0502	Rattus norvegicus NAD(P)H quinone dehydrogenase 1 (Nqo1), mRNA [NM_017000]
A_43_P12868	Smpd3	sphingomyelin phosphodiesterase 3	0.60	0.0000	0.0768	Rattus norvegicus sphingomyelin phosphodiesterase 3 (Smpd3), mRNA [NM_053605]
A_44_P137448	Ptgs1	prostaglandin-endoperoxide synthase 1	0.61	0.0000	0.0502	Rattus norvegicus prostaglandin-endoperoxide synthase 1 (Ptgs1), mRNA [NM_017043]
A_43_P12437	Hhex	hematopoietically expressed homeobox	0.61	0.0017	0.2996	Rattus norvegicus hematopoietically expressed homeobox (Hhex), mRNA [NM_024385]
A_44_P365286	Aldh1a1	aldehyde dehydrogenase 1 family, member A1	0.67	0.0003	0.2006	Rattus norvegicus aldehyde dehydrogenase 1 family, member A1 (Aldh1a1), mRNA [NM_022407]
A_44_P1052607	Aox1	aldehyde oxidase 1	0.71	0.0001	0.0924	Rattus norvegicus aldehyde oxidase 1 (Aox1), mRNA [NM_019363]

ProbeName	Gene Symbol	GeneName	log2FC	p-value	q-value	Description [target ID]
A_42_P761436	Npw	neuropeptide W	0.71	0.0006	0.2332	Rattus norvegicus neuropeptide W (Npw), mRNA [NM_153294]
A_44_P777328	Pik3r3	phosphoinositide-3-kinase regulatory subunit 3	0.72	0.0020	0.3025	PREDICTED: Rattus norvegicus phosphoinositide-3-kinase regulatory subunit 3 (Pik3r3), transcript variant X2, mRNA [XM_017593605]
A_42_P811256	Vnn1	vanin 1	0.74	0.0018	0.3025	Rattus norvegicus vanin 1 (Vnn1), mRNA [NM_001025623]
A_44_P182221	Gstp1	glutathione S-transferase pi 1	0.76	0.0000	0.0556	Rattus norvegicus glutathione S-transferase pi 1 (Gstp1), mRNA [NM_012577]
A_42_P791677	Areg	amphiregulin	0.81	0.0004	0.2006	Rattus norvegicus amphiregulin (Areg), mRNA [NM_017123]
A_43_P13342	Gstp1	glutathione S-transferase pi 1	0.90	0.0000	0.0057	Rattus norvegicus glutathione S-transferase pi 1 (Gstp1), mRNA [NM_012577]
A_42_P698240	Ptgr1	prostaglandin reductase 1	0.97	0.0034	0.3520	Rattus norvegicus prostaglandin reductase 1 (Ptgr1), mRNA [NM_138863]
A_44_P115192	Slc5a5	solute carrier family 5 member 5	1.09	0.0031	0.3520	Rattus norvegicus solute carrier family 5 member 5 (Slc5a5), mRNA [NM_052983]
A_44_P315672	Gcg	glucagon	1.13	0.0035	0.3520	Rattus norvegicus glucagon (Gcg), mRNA [NM_012707]
A_44_P200539	Cbll1	Cbl proto-oncogene like 1	1.14	0.0049	0.3884	Rattus norvegicus Cbl proto-oncogene like 1 (Cbll1), mRNA [NM_001108018]

P-value, OddsRatio and ExpCount: p-value, odds ratio and expected count based on hypergeometric test (GOstats R package). Count, Size: number of genes among the upregulated genes or in the full dataset respectively.

Table 4: Gene Ontology (GO) biological processes enriched (p<0.01) among the genes that are downregulated in the thyroid gland of female rats exposed to fipronil

GOBPID	Pvalue	OddsRatio	ExpCount	Count	Size	Term
GO:0006959	0,000004	26,41	0,25	5	54	humoral immune response
GO:0006957	0,000064	457,00	0,01	2	3	complement activation, alternative pathway
GO:0002684	0,000067	7,47	1,49	8	316	positive regulation of immune system process
GO:0002252	0,000132	7,75	1,21	7	256	immune effector process
GO:0072376	0,000226	30,98	0,12	3	26	protein activation cascade
GO:2000427	0,000317	114,19	0,03	2	6	positive regulation of apoptotic cell clearance
GO:0050729	0,000678	20,64	0,18	3	39	positive regulation of inflammatory response
GO:0009617	0,000772	6,61	1,15	6	244	response to bacterium
GO:0002460	0,000926	10,78	0,45	4	95	adaptive immune response based on somatic recombination of immune receptors built from immunoglobulin superfamily domains
GO:0002891	0,000941	57,05	0,05	2	10	positive regulation of immunoglobulin mediated immune response
GO:0050776	0,001014	6,26	1,21	6	257	regulation of immune response
GO:0032653	0,001147	50,70	0,05	2	11	regulation of interleukin-10 production
GO:0010827	0,001160	16,90	0,21	3	45	regulation of glucose transport
GO:0032101	0,001614	5,68	1,33	6	281	regulation of response to external stimulus
GO:0001934	0,002090	4,72	1,91	7	404	positive regulation of protein phosphorylation
GO:0050829	0,002164	35,08	0,07	2	15	defense response to Gram-negative bacterium

GO:0002712	0,002466	32,57	0,08	2	16	regulation of B cell mediated immunity
GO:0048584	0,002474	3,67	3,81	10	808	positive regulation of response to stimulus
GO:0030100	0,002637	7,99	0,59	4	126	regulation of endocytosis
GO:0008645	0,002799	12,20	0,29	3	61	hexose transport
GO:0032655	0,003484	26,80	0,09	2	19	regulation of interleukin-12 production
GO:0008643	0,003974	10,71	0,33	3	69	carbohydrate transport
GO:0050766	0,003990	24,82	0,10	2	22	positive regulation of phagocytosis
GO:0006911	0,004254	23,97	0,10	2	21	phagocytosis, engulfment
GO:0030490	0,004666	22,77	0,10	2	22	maturation of SSU-rRNA
GO:0002703	0,005027	9,81	0,35	3	75	regulation of leukocyte mediated immunity
GO:0043207	0,005092	4,44	1,67	6	353	response to external biotic stimulus
GO:0045937	0,005600	3,89	2,27	7	481	positive regulation of phosphate metabolic process
GO:0031348	0,005814	9,28	0,37	3	79	negative regulation of defense response
GO:0010324	0,006489	18,96	0,12	2	26	membrane invagination
GO:0006954	0,007297	6,02	0,80	4	198	inflammatory response
GO:0071674	0,007504	17,50	0,13	2	28	mononuclear cell migration
GO:0051251	0,007845	8,29	0,42	3	88	positive regulation of lymphocyte activation
GO:0032270	0,008052	3,35	3,07	8	650	positive regulation of cellular protein metabolic process
GO:0002708	0,008585	16,24	0,14	2	30	positive regulation of lymphocyte mediated immunity
GO:0016072	0,008604	8,00	0,43	3	91	rRNA metabolic process

GO:0002821	0,009151	15,68	0,15	2	31	positive regulation of adaptive immune response
GO:0001818	0,009405	7,73	0,44	3	94	negative regulation of cytokine production

Pvalue, OddsRatio and ExpCount: p-value, odds ratio and expected count based on hypergeometric test (GOstats R package). Count, Size: number of genes among the upregulated genes or in the full dataset respectively.

Table 5: Gene Ontology (GO) biological processes enriched (p<0.01) among the genes that are upregulated in the thyroid gland of female rats exposed to fipronil.

GOBPID	Pvalue	OddsRatio	ExpCount	Count	Size	Term
GO:0097756	0,000025	12,24	0,60	6	48	negative regulation of blood vessel diameter
GO:0035296	0,000050	8,42	0,99	7	79	regulation of tube diameter
GO:0050880	0,000069	7,97	1,04	7	83	regulation of blood vessel size
GO:0006880	0,000155	Inf	0,03	2	2	intracellular sequestering of iron ion
GO:0045987	0,000381	27,44	0,15	3	12	positive regulation of smooth muscle contraction
GO:0003100	0,000460	162,42	0,04	2	3	regulation of systemic arterial blood pressure by endothelin
GO:0007169	0,000479	4,06	2,85	10	228	transmembrane receptor protein tyrosine kinase signaling pathway
GO:0098869	0,000569	8,43	0,69	5	55	cellular oxidant detoxification
GO:0003056	0,000912	81,19	0,05	2	4	regulation of vascular smooth muscle contraction
GO:0007213	0,000912	81,19	0,05	2	4	G-protein coupled acetylcholine receptor signaling pathway
GO:0098754	0,000919	7,51	0,76	5	61	detoxification
GO:0007166	0,001051	2,46	10,55	21	843	cell surface receptor signaling pathway
GO:0007267	0,001150	2,81	6,31	15	504	cell-cell signaling
GO:0090066	0,001180	3,58	3,21	10	256	regulation of anatomical structure size
GO:0097237	0,001227	7,01	0,81	5	65	cellular response to toxic substance
GO:0007052	0,001331	9,51	0,49	4	39	mitotic spindle organization
GO:0051238	0,001508	54,12	0,06	2	5	sequestering of metal ion

GO:0030168	0,001761	8,75	0,53	4	42	platelet activation
GO:0071320	0,001761	8,75	0,53	4	42	cellular response to cAMP
GO:0002244	0,002096	8,31	0,55	4	44	hematopoietic progenitor cell differentiation
GO:1903831	0,002244	40,58	0,08	2	6	signal transduction involved in cellular response to ammonium ion
GO:1905145	0,002244	40,58	0,08	2	6	cellular response to acetylcholine
GO:0051384	0,002252	4,28	1,84	7	147	response to glucocorticoid
GO:0051716	0,002353	2,06	30,88	43	2466	cellular response to stimulus
GO:0034284	0,002432	4,21	1,87	7	149	response to monosaccharide
GO:0006979	0,002436	3,22	3,53	10	282	response to oxidative stress
GO:0002685	0,002926	5,67	0,99	5	79	regulation of leukocyte migration
GO:0002523	0,003116	32,46	0,09	2	7	leukocyte migration involved in inflammatory response
GO:0032355	0,003248	4,57	1,46	6	117	response to estradiol
GO:0001525	0,003579	3,53	2,54	8	203	angiogenesis
GO:0072358	0,004004	2,99	3,78	10	302	cardiovascular system development
GO:0030320	0,004121	27,04	0,10	2	8	cellular monovalent inorganic anion homeostasis
GO:0065007	0,004403	2,32	48,02	58	3835	biological regulation
GO:0019229	0,004537	10,15	0,34	3	28	regulation of vasoconstriction
GO:1900543	0,004919	9,85	0,35	3	28	negative regulation of purine nucleotide metabolic process
GO:0003013	0,005070	3,32	2,69	8	215	circulatory system process
GO:0002376	0,005208	2,17	10,39	19	830	immune system process

GO:0030010	0,005398	6,26	0,71	4	57	establishment of cell polarity
GO:0016477	0,005655	2,40	6,70	14	535	cell migration
GO:0000226	0,005929	3,54	2,19	7	175	microtubule cytoskeleton organization
GO:0007613	0,006105	6,03	0,74	4	59	memory
GO:0043200	0,006450	4,65	1,19	5	95	response to amino acid
GO:0032501	0,006493	1,89	31,28	42	2498	multicellular organismal process
GO:0051953	0,006516	20,28	0,13	2	10	negative regulation of amine transport
GO:1903018	0,006571	8,79	0,39	3	31	regulation of glycoprotein metabolic process
GO:1990266	0,006571	8,79	0,39	3	31	neutrophil migration
GO:0048869	0,006786	1,91	19,08	29	1524	cellular developmental process
GO:0048678	0,006870	5,81	0,76	4	61	response to axon injury
GO:0072676	0,007187	8,48	0,40	3	32	lymphocyte migration
GO:0048534	0,007446	2,71	4,13	10	330	hematopoietic or lymphoid organ development
GO:0007612	0,007697	5,61	0,79	4	63	learning
GO:0051049	0,007778	2,08	10,78	19	861	regulation of transport
GO:1901698	0,007872	2,19	8,45	16	675	response to nitrogen compound
GO:0001702	0,007900	18,02	0,14	2	11	gastrulation with mouth forming second
GO:0030818	0,007900	18,02	0,14	2	11	negative regulation of cAMP biosynthetic process
GO:0006811	0,007903	2,24	7,70	15	615	ion transport
GO:0010817	0,008153	3,04	2,92	8	233	regulation of hormone levels

GO:0006801	0,008517	7,93	0,43	3	34	superoxide metabolic process
GO:0009725	0,008554	2,28	7,02	14	561	response to hormone
GO:0001568	0,008779	2,80	3,58	9	286	blood vessel development
GO:0051234	0,008887	1,84	23,08	33	1843	establishment of localization
GO:0051674	0,009090	2,26	7,07	14	565	localization of cell
GO:0010648	0,009150	2,33	6,35	13	507	negative regulation of cell communication
GO:0023057	0,009150	2,33	6,35	13	507	negative regulation of signaling
GO:0030800	0,009404	16,21	0,15	2	12	negative regulation of cyclic nucleotide metabolic process
GO:0051225	0,009984	7,45	0,45	3	36	spindle assembly

Pvalue, OddsRatio and ExpCount: p-value, odds ratio and expected count based on hypergeometric test (GOstats R package). Count, Size: number of genes among the upregulated genes or in the full dataset respectively.