Plan Écophyto II – Appel à projets de recherche PNRPE

Programme « **2016** »

Écophyto Plan II - Call for research projects PNRPE

Program **"2016"**

« ACRONYME ET NOM COMPLET DU PROJET »

GePeTho : Gestation, Pesticides & Thyroïde

Effets sur la fonction thyroïdienne materno/fœtale d'une exposition à un mélange de pesticides contaminants la pomme : approche intégrative de l'exposition aux modes d'actions

Effects on materno-fetal thyroid function of an exposure to an apple pesticide mixture: integrative approach from exposure to the evaluation of the mode of action

0 – Ecophyto plan references

Number and description of the Écophyto II action the project refers to:

Axis **2 IMPROVING KNOWLEDGE AND TOOLS FOR TOMORROW AND ENCOURAGING RESEARCH AND INNOVATION**

Action **8 FOSTERING, ORIENTING AND COORDINATING RESEARCH PROJECTS TO PROMOTE COOPERATION BETWEEN MULTIDISCIPLINARITY AND COOPERATION BETWEEN ALL PLAYERS**

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Key words (5 maximum):

Orchard pesticide mixture - Thyroid disruption – Gestation - Fetal toxicokinetic -Low doses

1 – Recipient of the requested grant - identity

Beneficiary from the agreement with ONEMA:

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Project partners (if repayment of all or part of the grant):

The terms of repayment of subsidies among the partners designated above must be clearly indicated in the financing plan.

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2 –In a nutshell

Les pesticides à usage phytosanitaire constituent des sources majeures de contamination des aliments et de l'eau pouvant potentiellement affecter les systèmes endocriniens. Les données sur les propriétés de perturbateurs endocriniens des pesticides sont très insuffisantes. En outre, bien qu'étroitement réglementés par molécules, les effets potentiels sur la santé des pesticides en mélange et à faibles doses sont encore mal appréhendés surtout dans un contexte de perturbation endocrinienne pour lequel on sait que la période du développement fœtale constitue une période d'extrême sensibilité. Une étude récente, réalisée par l'un des partenaires de ce projet montre qu'un mélange de pesticides, fréquemment utilisés sur les pommiers et retrouvés sur les pommes, induit chez des souris adultes des perturbations métaboliques potentiellement liées à un dysfonctionnement thyroïdien et ce, à de très faibles doses. L'objectif de ce projet est de caractériser sur le plan qualitatif (identification des modes d'action) et quantitatif (étude dose/réponse en pré-criblage sur un modèle de référence) les effets potentiels de ce mélange de pesticides sur la fonction thyroïdienne durant la gestation. Les résultats obtenus devraient fournir des bases scientifiques pour l'élaboration d'un schéma d'évaluation des pesticides perturbateurs endocriniens en accord avec les critères définis par l'Union Européenne.

3 –/ Project short summary

Due to their extensive uses in agriculture, pesticides constitute a major source of food and water contamination. Many pesticides can alter endocrine functions of animals. GePeTo is focused on pesticides that can interfere with the thyroid function, a critical function in terms of neural development in all classes of vertebrates. We seek to determine the potential of a mixture of 6 pesticides frequently used in orchards and commonly found on apples in southwestern France to actually expose the fetus as a very sensitive target to thyroid disruption and to affect the thyroid homeostasis of pregnant animals and their offspring. Our specific goals are: 1- evaluate the main toxicokinetic pesticide parameters determining fetal exposure and trans-placental exchanges in the sheep and human 2- based on results of objective 1 and the results of a screening dose/response study in an amphibian reference model, link a maternal exposure at thyroid disrupting levels to fetal exposure and materno-fetal thyroid alterations in mammals 3- Lay scientific bases for a comprehensive understanding of the mechanisms underlying thyroid disruption in mother and fetuses 4- Gain scientific insights for the evaluation of the relevance of the different model for the prediction of thyroid disruption in human by determining the similarities/divergences between reference models for regulatory purposes (xenopus tadpole and rodents) and a mammal model relevant to human (the sheep).Our ultimate goal is to provide scientific bases for a better scheme of evaluation of pesticides as potential thyroid disruptors in consistency with the EU criteria for the identification of endocrine disruptors in the field of plant protection product and biocides published in June 2016 (*http://ec.europa.eu/environment/chemicals/endocrine/index_en.htlm*).

4 –Background and scientific and technical issues

Identification of the mixture of interest

In a previous experiment, we assessed the metabolic consequences of a chronic dietary exposure of adult C57bl6 mice to a cocktail of 6 pesticides (table 1, boscalid, captan, chlorpyrifos, thiaclopride thiophanate and ziram) at very low dose (TDI) upon a 12-month period. They are usually recommended for the treatment of apple trees and frequently detected in various consumed apple samples). Those pesticides are all extensively used in orchards and vineyard and as thus might constitute major sources of surface water contamination. Ziram is very sable in the environment and was classified by INERIS as a high priority substance for the evaluation of the ecological quality of waters. It is noteworthy that all the considered pesticides are classified as toxic for aquatic organisms under the CLP categories H400 and/or H410 i.e. acute toxicity level 1 or chronic toxicity long term nefast effects. Data and benchmark doses are available regarding fish toxicity but only very almost none are available for other aquatic vertebrates such as amphibians.

Our results showed that adult male mice exposed to the mixture had a significantly higher body weight than non-exposed animals. This was associated with early glucose intolerance (after 4 months of exposure) and a fasted hyperglycemia and a hepatic steatosis. In females the same metabolic disruption than in males could be evidenced. Steatosis is the first step of the non-alcoholic fatty liver disease a syndrome associated with impaired TH signaling. **Jointly to the many evidences on the potential of those 6 pesticides as thyroid disruptors, those results lead us to focus the current project on the hypothesis that this mixture might behave as a thyroid disruptor.**
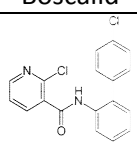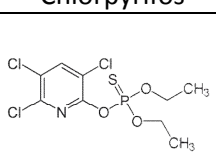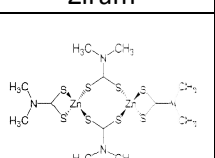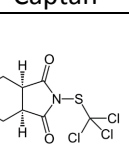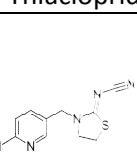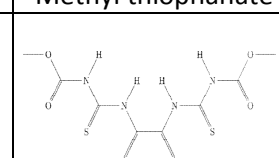
name	Boscalid	Chlorpyrifos	Ziram	Captan	Thiaclopride	Methyl thiophanate
formula						
Class	Carboxamid Nicotinamid	Organophosphate	Zinc-dimethyl-dithiocarbamate	Phthalimide	Neonicotinoid	Carbamate benzimidazole
TDI (mg/kg/d)	0.04	0.01	0.02	0.1	0.01	0.08
use	fungicide	insecticide	fungicide	fungicide	insecticide	fungicide

Table1: Pesticides of the mixture of interest.

Thyroid disruption: a public health issue

The incidence of thyroid pathologies keeps rising within human population with no clear explanation. One prevailing hypothesis is that environmental contaminants contribute to some degree to the deregulation of the thyroid axis. More and more data indicate that different classes of pesticides can interfere with the regulation of the thyroid function in experimental models. Among the 240 pesticides screened for carcinogenicity by the U.S. Environmental Protection Agency Office of Pesticide Programs in 1998, at least 24 (10%) produce thyroid follicular cell tumors in rodents [1].

Clinical hypothyroidism is a major causal factor for intellectual cognitive and motor retardation. Mild maternal thyroid alterations such as subclinical hypothyroidism (high TSH with normal free thyroxine (T4) or hypothyroxinemia (decreased free T4 without modifications of TSH), can be as well associated with neurocognitive disorders /deficiencies and/or modification of the central nervous system ontogeny. Contaminants might act during the fetal development of the central nervous system through the perturbation of the thyroid system of pregnant females and/or the developing fetuses [2,3].The functional consequences on behavior and neurocognitive competences of such alterations of the pattern of brain ontogeny are considered as major public health issues due to their elevated societal, affective and medical costs [4].

Which evidences for pesticides as thyroid disruptors?

In humans, evidences have accumulated for correlation between exposure to persistent pesticides such as DDT or chlordecone [5] and/or their metabolites as well as non-persistent ones in different setup (occupational, food exposure, developmental exposure..) and various degrees of thyroid disruption [6]. The biomonitoring surveys in human looking at the commonly used ethylene bisdithiocarbamates fungicides (EDBCs), to which **Ziram** belongs, are fully consistent with antithyroid, hypothyroid-like effect of ethylene thiourea evidenced in animal models. An investigation on a large size cohort of spouses of American pesticide applicators showed a significant increase of the odd ratios of the self-reported diagnosed hypothyroidism with ever use of some organochlorine insecticide, or the benzimidazole, dithiocarbamate fungicides [7]. Biomonitoring data examining specifically the link between **chlorpyrifos** exposure and thyroid function, in particular in the NHANES cohort, are consistently suggestive of an antithyroid effect of this organosphosphate insecticide [8,9]. Although harmonious thyroid function is critical for the development of the central nervous system during fetal life, biomonitoring survey on pre and perinatal exposure to pesticides and thyroid outcomes of the newborn or young children remain extremely scarce. **This highlight the urgent need for integrative approaches for a comprehensive understanding of the potential interaction between pesticides and the thyroid function in particular during pregnancy/development in relevant model to human gestation and thyroid physiologies.**

Numerous experimental studies have shown the ability of pesticides to affect thyroid homeostasis [6]. For example, in rats, decreased T4 in response to chlorpyrifos exposure was observed [10] while long-term exposure to chlorpyrifos-methyl induced hypothyroidism characterized by reduced T4 and increased TSH [11]. Exposure to chlorpyrifos, carbofuran, and dimethoate decreased serum T4 in ewes [12]. Ziram in rats exhibits antithyroid effect [13]. Captan exposure is associated to thyroid hyperplasia in rats pretreated with carcinogens [14].

Experimental data indicate that virtually all aspects of the thyroid economy can be affected by pesticides [15–17]. Carbamates carbaryl and naphtol metabolite showed thyroid antagonist activity *in vitro*, acting as disruptors of the TH receptor signaling pathway [18]. The main degradation product of many of the ethylene bisdithiocarbamates is ethylene thiourea, which is an antithyroid compound known to block thyroid peroxidase [19](TPO). TPO is the key enzyme of thyroid hormone biosynthesis responsible of iodine incorporation to the tyrosine residues of the stock protein, thyroglobulin (TG), to produce the two main thyroid hormones (TH), tri- and tetra-iodothyronine (T3, T4). In vitro,

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Ziram is effective to block TPO at 5µM. The sodium/iodide transporter (NIS, the cellular iodine transporter) activity, and iodide uptake by the thyroid glands are also potential targets for several pesticides including Ziram. The most recent available *in vitro* data on transfected cells expressing the human NIS however suggest that quite high concentrations (EC_{80} for iodide uptake inhibition>µM) are required for this effect to occur [20].

One of the most commonly reported mechanism of action is an effect of the pesticides on the hepatic catabolic rate of TH. Induction of hepatic phase II enzyme and/or TH cellular phase III transporter has been shown in response to several pesticides and related to thyroid disturbances. Boscalid is acknowledged as a secondary thyroid disruptor acting through hepatic catabolism [21]. Studies performed by partner 1 (Toxalim Team 1) showed that fipronil, a broad-spectrum phenylpyrazole insecticide, is associated to an increased clearance of T4 due at least in part to the overexpression and increased activity of UGT enzyme conjugating TH [22–25]. Numerous pesticides are ligands of xenosensing nuclear receptors (XNR). XNRs are transcription factors able to induce phase I and II xenobiotic metabolizing enzymes as well as transporters (phase III) including TH-related (UGT, SULT, OATP, deiodinases) and energy metabolism pathways, laying the foundations for potential crosstalk between pesticides and TH physiological functions including energy expenditure regulation. **Liver-mediated thyroid disruption is usually assumed to be non-relevant to human, although there is currently no available proper quantitative assessment of this assumption.**

Evaluation of thyroid disruptors :

In OECD (*OECD series on testing & assessment, 2006, n°57, 434 p)* and US EPA endocrine screening programs, several integrated models are used for thyroid investigation, in a tier and weight of evidence approach, in which the detection of anti-thyroid chemicals is conducted by *in vivo* tests exclusively. Amphibian metamorphosis assay (OECD TG 231, first step) includes thyroid gland histopathology as first step of screening. The Xenopus Embryonic Thyroid Assay (XETA) assay provided by the CNRS Partner is currently under validation process (phase 2) and permits within a 3-day time frame to decipher whether the substances, alone or in mixture alter the thyroid hormone axis.

For mammals, despite a permanent debate on the relevance of rodent models toward human thyroid physiology, the only *in vivo* proposed OECD guidelines for thyroid are rodents screening assays (*OECD series on testing & assessment, 2012, n°150, 524 p*). Thyroid investigations in those tests are limited and are not specifically designed for thyroid disruption. They are included in male and female pubertal assays, described by US EPA (*US EPA OPPTS 890 1450-1500*), and other repeated doses toxicity assays (OECD TG 407, 416, 443). In all these assays, histopathology and serum hormone changes are considered as the key-endpoints for thyroid-related activity. However, the guidelines recommend the non-quantitative histopathology assessment and only one or two sections of the gland are routinely analyzed, limiting the sensitivity of those endpoints. Hormonal changes (T4, T3, TSH) are evaluated at a given time point, usually at animal euthanasia with no consideration of physiological, environmental or experimental factors that can affect TH concentrations (temperature, stress, stage of the estrous cycle…). It is usually assumed that histological parameters such as the follicle size, height of epithelium are good surrogate of TSH-dependent hyperactivity of the thyroid gland and an alert signal for neoplasia development in rodents. This however might not hold true for humans. TSH levels in humans are indeed much lower than in rodents [26] suggesting that the thyroid gland might not have the same TSH requirement as rodents. In human, the follicle size is large and the colloid is abundant, asserting the huge capacity of the gland as a reservoir of TH. By contrast, in rodents the follicle size is rather small and the colloid is much less abundant consistently with a high cellular and metabolic rate and higher predisposition to hyperplasia and consecutive neoplasia.

The regulatory evaluation of the thyroid function in mammal models is extremely limited and conducted in a model that is permanently challenged for its relevance to human thyroid physiology. Despite that there is absolutely no recommendation for using other models and therefore very few data for a proper evaluation of interspecies differences, that could serve as a base for extrapolation of experimental animal data to human.

5 – The project

Detailed description of the objectives

Thyroid homeostasis is a privileged target for numerous pesticides acting through a whole panel of mechanisms including thyroid and non-thyroid targets such as the liver. Those alterations of thyroid function when they occur during fetal development might induce lifelong deleterious consequences in particular in terms of neurocognitive capacities. The weaknesses of the scientific scheme of evaluation of thyroid disruptors is aggravated by the absence of recommended specific tests for in depth investigation of thyroid function during the gestation, a window of extreme sensitivity to endocrine disruption. Finally the numerous controversies regarding the relevance of the animal models,

in particular rodents, toward human thyroid physiology contribute to divide the scientific community and add to the general confusion. The ultimate objective of the GePeTho project is to lay the scientific foundations for a fully integrative scheme of evaluation of thyroid disruptor pesticides during gestation taking into account both toxicokinetic considerations relative to fetal exposure and mechanistic considerations on the signaling pathways underlying thyroid disruption. We propose a quantitative assessment for several parameters characterizing the thyroid axis functionality as premises to normative approaches. This will be achieved through four specific objectives:

1- Evaluate the main toxicokinetic pesticide parameters determining fetal exposure and trans-placental exchanges in the sheep and human

2- Based on results of objective 1 and the results of a screening dose/response study in an amphibian reference model, link a maternal exposure at thyroid disrupting levels to fetal exposure and materno-fetal thyroid alterations in mammals

3- Lay scientific bases for a comprehensive understanding of the mechanisms underlying thyroid disruption in mother and fetuses

4- Gain scientific insights for the evaluation of the relevance of each model for the prediction of thyroid disruption in human by determining the similarities/divergences between reference models for regulatory purposes (xenopus tadpole and rodents) and a mammal model relevant to human (the sheep).

5-

Pioneering nature of the project

- "Real life mixture": low doses, molecules frequently encountered in the south west of France
- Integrative approach in different animal vertebrate models, each of them being carefully chosen for their respective interest regarding the different regulatory events and/or signaling pathways of the thyroid function, scientific data to address the critical question of the relevance of animal models
- Quantitative approach/ Premise for normative evaluation
- The project targets fetal development known to be a critical window of sensitivity to thyroid disruptor pesticides.

Interest in view of the Écophyto Plan

- The mixture in focus has been identified in a fruit widely consumed by all classes of the population (apples)
- Real impact on the process of hazard identification in conditions relevant to human exposure (molecules-mixture -low doses- assessment of animal model relevance)
- Establish different indicators for the impact of pesticides on the thyroid function during gestation. Identification of the most sensitive and selective ones
- Establish the most relevant model/approach for the risk analysis for human health in terms of thyroid disruption: a more adequate evaluation = decreased level of uncertainty; this should accelerate the process of evaluation.
- Recommendation relative to the exposure of a very sensitive population (pregnant women).

Structuration and methods

WP 1 : Organization management :

Consortium agreement :
- Consolidated Final budget
- Calendar
- Dedicated Page web on Toxalim webpage
- Publication strategy –authorships
- Meetings : kickoff-midterm to prepare the mid-term report
- Creation and access to a shared single budget file (in coordination with the budget secretary of each team)

months

Worpackage	Tasks	Partners - Teams	Delivrables
1-Managment	Meetings Agreements…	all- lead P 1	Consortium agreement - ethical requirements animal experimentation)- meetings schedule
2-Developmental exposure scheme: dose screening- fetomaternal toxicokinetic- relevance to	Evaluation of thyroid-disrupting effect in tadpole: dose -response relation ship of the total mixture	P 2 CNRS/MNHN	Identification in tadpole of the minimum doses with a thyroid effect. Determination of exchange constants between the mother and the fetus in the sheep: dose to be used in the sheep
	Fetomaternal toxicokinetic in the pregnant ewe	P1 Team3-7	
	Determination of exchanges rates through perfused human placenta	P1 Team3-7	Relevance to human in terms of potential fetal exposure
3-Calibration of the histological morphometric approach	PTU-induced hypothyroidisms- calibration of histological modifications // to changes in blood concentrations	P3: CRFRE P1 team 3	Modeling the relation between different thyroid morphometric parameters and TH concentrations. Standardized procedure for thyroid histological evaluation and gradation of thyroid disruption.
4-Assessment of the "mixture effect" on the tadpole	Mixture effect vs simple exposure : full scale dose/response stydy	P2: CNRS/MNH	Quantitative assessment of the mixture effect vs simple exposure, determination of a model characterizing the mixture effect.
	Thyroid histological modifications	P3: CREFE	
	Thyroid hormones assay	P1: Team 3	
	Scaling TH concentration/ thyroid histology parameters	P1-P2-P3	
5- Assessment of the liver-thyroid axis in rodents	Animal treatments	P1 Team1	Caracterisations of the thyroid disrupting properties of the mixture in the mouse sensitive reference model in pregnant animals. is the hepatic catabolism of thyroid hormones a privilegied target ?
	Thyroid histology mothers	P3: CREFRE	
	TH hormones mothers and pups	P1 Team3	
	thyroid-related Hepatic biomarkers mothers and pups	P1 team 1	
6-Sheep developmental exposure : in depth investigation of the mode of action on the thyroid axis including hepatic clerance of TH	animal exposure and preparation	P1 team 3	In depth investigation of possible modes of action of the mixture on the whole thyroid axis 'hypothalamo pituitary-thyroid-blood transportation-hepatic catabolims) in the human-relevant sheep model. Convergences/ divergences with the two other models as a base for examining the relative relevance of each model toward human.
	Thyroid axis pharmacological investigation (TRH TSH) T4 clearance in mothers	P1 teams 3 - 7	
	Maternal/newborn thyroid histology (one lobe)	P3: CREFRE	
	TH TSH hormones measurment	P1 team3	
	Thyroid expression and/or activity of key regulators of TH biosynthesis (TPO, thyroglobulin, deiodinase, NIS , pendrin)	P1 team3	
	TH binding capacities in treated animals	P1 team3	
	Impact of the mixture on TH plasma binding capacity in vitro	P1 team3	
	Liver regulators of thyroid homeostasis (TBG TTR deiodiase UGT SULT OATP MCT..) + hepatic targets modified in rodents	P1 teams1-3	
	Publication		
	Meetings		

Final report

Partner n° / ID	Team	
n°2 CNRS/MNHN	Evolution of Endocrine Regulations	
n°1: INRA Toxalim	1-Integrative Toxicology and Metabolism	
	3-Gestation &Endocrine Disruption	
	7-Pharmacokinetic-Pharmacodynamic-Modeling	
n°3: ENVT	CREFRE	

WP 2Developmental exposure scheme: functional screening for thyroid disruption in the tadpole model- feto-maternal toxicokinetic in the ewe- human trans-placental exchanges

2.1 Dose/response relationship of the mixture in tadpole

Methods

The actual XETA gene reporter assay is powerful thanks to its rapidness and its easy readout (fluorescence quantification in GFP-transfected tadpoles with the GFP gene under the control of the thyroid responsive element in the brain). XETA is sensitive and allows detection of chemicals wherever the chemicals act on the thyroid hormone axis (synthesis, iodine intake, distribution in blood stream, membrane transporters, at the receptor level). Mixture of the 6 pesticides found on apples will be tested in the XETA system at five different concentrations: actual concentration found in apples, TDI for each individual substance and at higher non-toxic concentrations in order to establish a full scale dose/response curve and, with or without T3 (agonistics or antagonistic effects). The result will be used along with toxicokinetic data generated by partner 1 to refine the scheme of exposure in mammals.

Pitfalls

One can argue that tadpole might not be relevant to mammals. It has to be emphasized however that it is a reference model to screen for thyroid disruption in regulatory toxicology. This is also a convenient, fast and sure model allowing to perform a full scale dose/response relationship which would be too time and animal consuming in mammal. In addition it has to be emphasized that partner 1 (Toxalim's team 1) already generated data unambiguously showing that the mixture at very low level (TDI) can elicit endocrine-related deleterious effect in adult mouse that are very likely to be at least in part thyroid-mediated.

It will not be possible to identify modes of action. In order to add mechanistic insights, some additional readouts could be done based on what will be observed in mammals.

2.2 Evaluating sheep fetal exposure from maternal treatment

The sheep model for fetal exposure
The extent and time course of drug fetal exposure is the result of a complex interplay between the kinetics of placental drug transfer as well as many other factors related to maternal and fetal components, including maternal and fetal clearances, maternal to fetal and fetal to maternal placental clearances and the recirculation of drugs between fetal fluid compartments. The pregnant ewe is an acknowledged model for studying nutrient exchange [30] and is frequently used to characterize drug disposition during the prenatal period [31,32]. The model of chronically *in utero* catheterized ovine fetus, already developed by team GED of Toxalim, allows serial sampling of maternal and fetal blood, as well as other fluids, after maternal or fetal dosing [33].

Specific objectives
The objective of this task is to determine the main toxicokinetic parameters characterizing feto-maternal exchanges of individual substances after mixture administration to pregnant ewes. This will be used to determine the dosing scheme in the ewe that will produce in fetal plasma drug concentrations within the range of those that will have been associated to thyroid disruption in xenopus tadpoles.

Methods:
The time course of drug concentrations in maternal and fetal plasma will be determined after separate maternal (n=5) and fetal intravenous administrations (n=5) of the mixture. A two-compartment open model with drug elimination occurring from both the maternal and fetal compartments will be selected to fit drug concentrations simultaneously in fetal and maternal plasma and compute the maternal and fetal placental and non-placental clearances. The mixture will also be administrated in food to 5 sheep in order to evaluate the bioavailability for this route of administration.

Expected results
The compartment model will be used to predict for each of the component of the mixture, the doses and frequencies of maternal administration required to maintain the targeted plasma fetal concentrations. These data along with oral route bioavailability will be used to determine the experimental scheme of pesticides administration to pregnant ewes via the food.

Pitfalls and limitation
Performing surgical *in utero* catheterization of fetus requires special skills. The success of this method is guaranteed by the expertise of partner 1 (Toxalim's team3). The toxicokinetic parameters will be determined on a single administration paradigm in a late stage of gestation while in task 5 the animals will be chronically exposed throughout

gestation. One cannot exclude that toxicokinetic might at some point differ from the acute one at a late stage and therefore lead to different exposure than the model-predicted ones. This will be in part controlled through measurement of toxicant blood concentrations in the pregnant ewes and if necessary adjustment of the treatments.

2.3 Evaluation of the relevance of fetal exposure scheme/human placenta physiology

A better knowledge of the transplacental permeability in human is required in order to provide a reliable basis for extrapolating the risks caused by drug exposure, from animal models to humans. The *ex vivo* human placental perfusion model represents a gold standard for evaluating the placental drug exchanges in humans.

Specific objective

The objective of this task is to evaluate in an open (non-recirculating) human cotyledon, the placental transfer of the components of the mixture and determine their maternal and fetal placental and non-placental clearance values. Studies will be performed on 10 isolated human placentas using the method previously described [34]. Human placentas will be obtained from pregnant mothers undergoing programmed caesarean or spontaneous deliveries.

Expected results

The maternal-to-fetal and fetal to maternal placental clearances will be calculated from the steady state drug concentrations evaluated in each of the receiving and entrance compartments.

These values will be used to scale toxicokinetic model developed in sheep to predict human fetal exposure.

Pitfalls and limitation

The placental perfusion method is based on an open (non-recirculating) system. This single pass system is a metabolically static system that does not enable placental metabolism to be evaluated. *Ex vivo in vitro* approaches can be performed to evaluate the placenta metabolic capacities and mechanisms regarding the mixture molecules.

WP-3: Is the morphometry of thyroid a sensitive biomarker of (sub) hypothyroidism

In rodents, the exquisite sensitivity of the thyroid to the stimulatory effect of TSH on cell dynamic can constitute an interesting sensitive parameter to evaluate and quantify the potential of a drug to alter thyroid function through TSH modifications. The structural characterization of the thyroid is a very valuable endpoint to assess functional changes in many species [35].

Objectives

The aim of the calibration study in mice is to demonstrate that histological endpoints can be used as early sensitive biomarkers of thyroid disruption, with higher sensitivity than circulating T3, T4 and/or TSH. The specific objective is to develop a TH concentrations/response approach by modeling the quantitative relationship between TH and TSH blood concentrations in relation to different key morphometric parameters of the thyroid gland in PTU-induced hypothyroidism.

Methods

Eight week old C57BalbC mice (lower level of expression in TBG) will be treated for 30 days with PTU in drinking water (8-10 male mice/group). The highest dose will induce a significant decrease of T3 and T4 and increased TSH (clinical hypothyroidism); 4 other lower doses will be used to induce different levels of hypothyroxinemia associated or not to TSH modifications. At termination, serum and the thyroid will be collected for TH, TSH assays and histology, respectively.

Quantitative parameters characterizing the thyroid functionality will be generated using computerized image analysis (Lucia ® software, Nikon, France) on serial 3 µm thin coronal sections of the thyroid gland (1 section every 100µm) after both hematoxylin eosin and Schiff periodic acid colorations. This will include in particular number and distribution by size classes of the follicles, height of thyrocytes and size of the lumen, incidence of vacuoles in the thyroglobulin, immune histochemistry expression of markers of cell multiplication.

Expected results

The thyroid being the ultimate step of integration of all the regulatory processes of the thyroid axis, this study should provide critical information to determine whether there is one or several thyroid morphometric parameters that provide a more reliable and sensitive biomarker of thyroid disruption than simple TH, TSH assay

It will be possible to determine which parameters are the best predictor of the subsequent modifications of TH blood concentrations. **The calibration scale that will be established will be used as a base for the comparisons with the other species of this project and will help to objectivize those interspecies differences that remain at the center of the controversy on the relevance of rodent model to human.**

WP-4: Assessing the mixture effect vs single molecule exposure. Shape of the relationship between TH tissue concentration and morphometric parameter of the thyroid in tadpoles

Assessment of the mixture vs single molecule effect

The goal here is to address the question of the model followed by the mixture effects as compared to individual molecules. This is a critical question in particular in terms of ecotoxivological risk assessment. We will thus study single compounds thyroid disruptive potential in dose/response paradigm o. The shape of the curve and the main pharmacodynamics parameters (potency, efficacy) will be generated and comparde to the result in the mixture dose/response assay.

We will test the concentration at Tolerable daily intake (TDI) and 4 other non-toxic concentrations surrounding this concentration (10, 100-fold higher and 10,100-fold lower) with 72h exposure in the presence and absence of T_3 for each of the 6 targeted pesticides on our TH reporter (TRE- GFP) xenopus (XETA) on stage NF45 tadpoles in presence of absence of T3 at 5nM

All the active concentrations and the highest non-active one of either single compounds or the mixture will be tested on naïve animals. The same protocol than the one used for the XETA will be used (3 day exposure with daily renewal). After exposure, animals will be collected in order to assay TH (T3 and T4 ad minima) in the tadpoles using HPLC/MS on pools of 60 to 90 tadpoles. A morphometric analysis of thyroid gland will be performed by the CREFRE team according to the method developed and validated in mouse. Alike in the mouse model, the relationship between TH tissue concentration and thyroid morphometric parameters will be evaluated. This will allow to determine if this relation follows the same rules in between the two species.

Expected results
The single compound approach will strengthen the results obtained with the mixture. TH tissue concentrations will be measured (Partner 1 Toxalim's team 3) to provide insights on the mechanism of action of these compounds and will be put in perspective of thyroid gland histology as a new endpoint for possible biomarker using the scaling approach developed by partner 3 (ENVT: CREFRE) in mouse.

WP-5 Rodent developmental exposure: the liver a privileged target mediating thyroid disruption

The rodents are acknowledged as a highly sensitive specie for liver-mediated thyroid disruption characterized by increased hepatic catabolism of thyroid hormone, one of the most frequent mode of action of thyroid disruptor pesticides In addition, the interactions between XNRs and energy expenditure have been largely investigated in rodents. Thus it can be considered that rodents are among the most appropriate models to elucidate in first instance the mechanistic pathways underlying the 3 partner cross-talk: liver XNRs –thyroid disruption- metabolic alterations.

Specific objective:

Our preliminary results show that dietary exposure upon a 52-week period of adult mice to a mixture of 6 pesticides at low doses led to significant metabolic disturbances in a gender dependent manner **suggesting an endocrine disrupting potential of the pesticide mixture**. It has been previously demonstrated that impaired TH signaling is associated with steatosis (Mullur R 2014) and that T3 can directly regulate liver lipid homeostasis. **Therefore, we hypothesize that thyroid disruption is affected in male mice upon pesticide exposure and that the liver might be a primary target for the pesticide mixture to induce thyroid disruption in our model.** This hypothesis is currently being checked following adult exposure.

In this project, we aim to assess the **transgenerational impact** of pesticide exposure during critical **periods of development** (gestation and lactation) on thyroid and metabolic homeostasis in descendants by **focusing on the liver-thyroid axis.**

Methods
Animal Feed Preparation Unit (UPAE, National Institute of Agricultural Research (INRA), Paris, France) will incorporate the mixture previously mixed in vitamins powder in classical rodent pellets. Two groups of 10 female mice will be fed with the pesticides enriched diet or the equivalent control diet from mating and during gestation and lactation. At weaning, 10 male and 10 female pups from exposed and non-exposed group will be weighted and sacrificed. Serum, liver and thyroid will be harvested from both the pups and the dams for TH, TSH blood concentration and thyroid histology, respectively.

Thyroid related hepatic biomarkers (target genes of nuclear receptors XNRs, CAR PXR PPAR, and TH receptors and more particularly those involved in TH economy such as TTR, TBG, deiodinases genes) in pups and mothers will be

analyzed by microarrays. In addition, hepatic changes related to metabolic disturbances previously evidenced in adult will be assessed by transcriptomic, metabolomics and biochemical approaches in liver and serum. All these parameters will be analyzed in conjunction with thyroid histological parameters and TH in blood. In half of the dams, the thyroid will be collected for qPCR analysis of the key factors of TH biosynthesis (NIS, TBG, TPO, TH transporters, pendrin, deiodinases..) and in the other half, the thyroid will be fixed for histological morphometric evaluation as developed and established by the CREFRE team (Partner3).

Expected results

1) Identification of liver and thyroid disturbances in pups will support (1) that gestation and lactation are critical windows of exposure to pollutants, (2) the impact of thyroid disrupting pesticides even at low doses on offspring.

2) Identification of metabolic interplay between liver and thyroid that leads to the dysregulation of metabolic homeostasis upon pesticide exposure.

Pitfalls and limitation:

Although the main focus of the project is on thyroid disruption and the mode of action of the pesticides on the thyroid function, other deleterious effects might be expected. Thus, other tissues will be collected and properly stored for further investigations in other projects. In particular, it will be interesting to perform in depth investigation on the potential mechanisms and actors of energetic metabolism disturbances if confirmed within the frame of the current developmental exposure. The microbiota of the offspring as a key regulator of energy balance could in particular reveal itself a very interesting and relevant endpoint to investigate in further projects. The brain of the offspring will be of tremendous interest in particular if maternal thyroid disruption is observed.

WP-6 Sheep developmental exposure : one step further toward human

Hypotheses and objectives

One of the main criticisms that are addressed to the rodent model is the absence and/or the expression of poorly efficient thyroxin Binding globulin (TBG) in the adult. In humans, as in some other mammal species, TBG is the most efficient TH binding protein. It largely contributes to protect TH from catabolism and provides a high "buffering capacity" toward all processes leading to increased elimination of TH. In addition, the dependency of the fetus toward TH maternal supply is particularly important at the early stages of the development. Fetal thyroid function starts only after the first third of the pregnancy in human as well as in sheep while it starts much later [36,37](last third of pregnancy in rodents). For these reasons, this is one of our main hypotheses that sheep might be more relevant to human than rodents for studying thyroid disruption proceeding from increased hepatic catabolism of TH during gestation.

The underlying hypothesis of this part of the project is that gestational exposure to pesticides will be associated to maternal and/or fetal disruption. Our specific goals are to characterize this disruption and to understand the underlying mode of action of the pesticides. To this aim, extensive functional investigations will be performed in two different groups of ewes (control and mixture treated 6 molecules in food at levels determined in WP2) at different level of regulation of the thyroid axis.
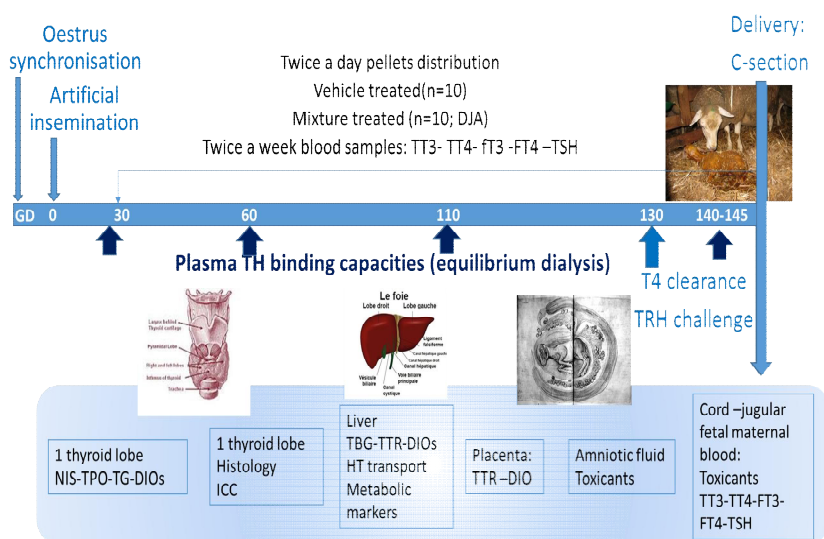
Methods

Critical TH-related gene expressions (NIS-TPO-Deiodinases DIOs, OATP, MCT transporters, TBG, TTR, UGT, SULF…) will be determined using qPCR and whenever possible at the level of protein expression using western-Blot analysis with infrared technologies (Odyssey®) for real quantitative assessment, or direct measurements of activity (NIS, TPO, DIO, UGT, SULF).

Thyroid hormone and TSH blood concentrations will be assayed using validated radioimmunoassays except for T4 clearance determination. T4 clearance determination will be performed by modelling the 24h time course of blood concentrations of ^{13}C-labelled T4 administered by the IV route. The assay

method for ^{13}C-T4 and its metabolites by HPLC/MS (no possible confusion with endogenous T4) will be developed by the analyst engineer of team 7 from Toxalim (partner1). The TRH challenge will be performed at 24h post ^{13}C-T4 administration, a time when the TSH response to T4 feedback reaches a maximum suggesting a seriously dampened secretion of endogenous TRH and thus TSH which should contribute to a better response to exogenous TRH with lesser inter-individual variability. Equilibrium dialysis [38] is mastered by partner 1 (Toxalim team 3 GED) and will be used to monitor both plasma TH binding capacities in treated animals and the impact *in vitro* of the mixture on these binding capacities in control animals.

Expected results
This approach addresses all levels of the thyroid axis regulation at least in the mothers and will take our investigation far beyond the hepatic level. At this level of the project we will have an idea of interspecies variability that we should be able to relate to the specific mode of action.

Pitfalls
No functional consequences of the thyroid disruption is addressed in the sheep. Indeed, given the peculiarities of energy metabolism regulation in polygastric animals it did not seem sound to us to assess this question in this model. Team 3 of Toxalim, successfully developed in collaboration with the platform AXIOM and D Zalko's team a metabolomics approach on specific cerebral structures to identify biomarker of fetal exposure to endocrine disruptors. Thus, brains will be collected and properly stored to be used for this type of investigation in further projects.

Control and monitoring rules of the project: partners, skills and human resources

Control and monitoring rules will be defined during the course of WP1 (see above) to build the consortium agreement.

Partner 1: UMR INRA Toxalim

Toxalim Team 3 : Gestation & Endocrine Disruption: This team is led by the coordinator of the project. Two professors from the National Veterinary school of Toulouse will lead all the aspects regarding fetal exposure in sheep and its relevance for human. This research area has been extensively and fruitfully developed in the team by V Gayrard and N Hagen-Picard for the last five years. C Viguié, DR2 INRA will ensure the coordination of all the tasks and development related to thyroid regulation and gestational exposure with the technical support or the laboratory technician, a study engineer and a PhD student who will be recruited for the project. C Viguié led a PNRPE project on the effect of fipronil on thyroid function using multispecies approaches including human that gave rise to 9 rank A publications The technical staff for animal husbandry will insure the daily animal care and treatment.

Toxalim Team 7: the main role of team 7 will be to drive TK analysis and modeling approaches. (Pr A Bousquet Mélou, Pr emeritus PL Toutain). As so, they will contribute to the setting up and realization of the experimental design of animal phases in set up allowing proper TK analysis and modeling. The team will also render available for the project its animal facilities and analytical materials and technical staff (one Research engineer) to fullfill the analytical needs for HPLC/MS Thyroid hormone assay and q-PCR analysis. The biomathematicians of the team will help with statistical analysis and modeling (1 CR2 INRA R Servien).

***Toxalim Team 1* Integrative Toxicology and Metabolism, led by Hervé Guillou is studying molecular mechanisms of hepatic physiopathology and regulation by by nutrients and xenobiotics. L Gamet-Payrastre** is interested in the impact of dietary exposure to low dose pesticides mixture and has coordinated 2 national projects supported by ANR and ANSES. She was involved in an international project PHC IMHOTEP to study the transfer of pesticide residues to mice offspring upon prenatal exposure and the ameliorating effect of anti-oxidant (Mansour et al 2013). She is coordinator of a regional project: NEWPOM (2016-2018) on the impact of pesticides mixture upon dietary or dermal exposure on hepatic metabolism

Partner 2: UMR7221 CNRS/MNHN
Evolution of Endocrine Regulations. The team is studying gene regulation in amphibian metamorphosis using fluorescent fusion proteins combined with transgenesis. This approach provides a versatile model for analyzing, within an integrated *in vivo* context, physiological TH-dependent regulations on specific tissues and cell types and is currently under OECD validation process. This technology which will be used in GePeTho to decipher thyroid disrupting effect of mixture and simple compounds led to the creation of the WatchFrog start-up (http://www.watchfrog.fr/) and the nomination of B Demeneix (PR MNHN, lead of the team) for the CNRS Innovation medal in 2014. of the The team recent research on seeking brain effects following embryonic exposure to

endocrine disrupting compounds, gave rise to more than 10 international invited conferences in 2016 from both JB Fini (CR CNRS) and B Demeneix. Both JB Fini an B demeneix will be deeply involved in the conduct of the project and they will be helped by the recruitment of a dedicated technician.

Partner 3: CREFE.

This team includes two professors from the National Veterinary School of Toulouse, one toxicologist (Martine Kolf-Clauw) and one histologist (Nathalie Bourges-Abella) working on the CREFRE (Regional Center of Functional Explorations) and on Histopathology plateform. Both have been working on the rodent model and on various animal tissues for many years, They will conduct the calibration pilot study in chemically induced hypothyroidism in mice, to precise and compare the sensitivity of the histomorphometric and hormonal endpoints, at different levels of thyroid functional disruption. They will have the technical support of Céline Bleuart, research technician specialized in histology. Given the fact that computerized image analysis can be highly time consuming in particular with large size samples an engineer assistants and tow master students will be hired.

Expected results, particularly in terms of reducing the use, associated impacts and risks from plant protection products

On the general point of view, the benefit of this project can be deciphered essentially in terms of improving the risk analysis for health of pesticides through a better consideration of thyroid disruption:

The integration of all the data in the three species should constitute a so far unique set of data to evaluate the respective relevance, benefits and limits of these models. This should constitute a valuable piece of information to refine the scheme of evaluation of pesticides regarding their potential as thyroid disruptors.

Our results should highlight the extreme sensitivity of pregnant women to thyroid disruption and should bring pioneer data for a better consideration of this class of the population in the conceptual frame for the evaluation of endocrine disruptor pesticides.

Expected deliverables and optimization of results for the benefit of Ecophyto

The recent definition of the EU for endocrine disruptor phytopharmaceutical substances published in june 2016 (a classification that can lead to the ban of the substances) mentions that the proof of the endocrine mode of action is absolutely required. As far as thyroid regulation is concerned, there is so far no clear set of criteria and no consensual models that will allow to conclude on a thyroid mode of action. Our results should definitely help to refine the scheme of evaluation of pesticides as thyroid disruptors according to the EU criteria. *https://www.efsa.europa.eu/fr/efsajournal/pub/4038*

Several of the proposed approaches are quantitative and as so could be used for normative regulation.

Finally, the use of the tadpole model is a good way to address the question of ecotoxicological assessment of pesticides with thyroid disruption properties on aquatic non-fish vertebrates.

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6 –Financial characteristics

The total cost of the project "*GePeTho*" is "*total project*" €: **934 248 €,**

including "*eligible amount*" € are eligible for a grant.: **340726 €,**

requested by the consortium: **246 421 €**

7 –Planned implementation schedule

Date de démarrage du projet : expected date : 2017

Durée prévisionnelle du projet : 36 mois – 36 months