ABSTRACT

FLIPPIN, JENNIFER LYNN. Predictive Modeling of Thyroid Hormone Disruption and Investigation of Potential Biomarkers of Chemical Exposure. (Under the direction of Kevin M. Crofton and Gerald A. LeBlanc.)

Humans and wildlife are exposed to thyroid hormone disrupting chemicals in their environments. These exposures often occur as complex mixtures of xenobiotics with similar and dissimilar modes of action. This research tested the hypothesis that serum thyroxine (T_4) concentrations in rodents exposed to a mixture of chemicals with two distinct modes of action (enhancers of T_4 hepatic metabolism and inhibitors of thyroidal hormone synthesis) could be best predicted by a model of integrated addition. Female Long-Evans rats, 23 days of age, were dosed with dilutions of a mixture of 18 polyhalogenated aromatic hydrocarbons (dioxins, dibenzofurans, and polychlorinated biphenyls) and a mixture of three pesticides (pronamide, mancozeb, and thiram) for four consecutive days. Serum was collected 24 hours after the last exposure and T_4 concentrations were measured by RIA. Animals exposed to the highest dose of the mixture experienced a 45% decrease in serum T_4. Three additivity model predictions (dose addition, effect addition, and integrated addition) were generated and compared. The response to the mixture was best modeled by integrated addition in which responses to the pesticides and polyhalogenated aromatic hydrocarbons were separately evaluated according to dose additivity, and the response of the two groups of chemicals were summed according to effect additivity. The response to the mixture was also well-predicted by a dose addition model. These results lead to a clearer understanding of thyroid hormone disruption by a complex mixture and show that

models are able to accurately predict the response of T_4 to a mixture of endocrine disruptors.

Thiram, mancozeb, and pronamide inhibit thyroid hormone production in the thyroid gland. These hormone decreases may be associated with altered expression of thyroidal genes. It was hypothesized that exposure to TH synthesis inhibitors would result in altered expression of mRNA of PAX-8, Hex, Tg, NIS, and/or TPO in the thyroid gland. Female Long-Evans rats were exposed for four days to two dilutions of each of the following mixtures: three pesticides (thiram, mancozeb, and pronamide); 18 PHAHs (dioxins, furans, and PCBs); a combination of the three pesticides and 18 PHAHs. Thyroids were collected and rtPCR was used determine if alteration of key thyroid genes occurred as a result of TH synthesis inhibition. Thyroidal expression of PAX-8, Hex, Tg, NIS, and TPO mRNA was not significantly different than corn oil treated control animals. The expression patterns of the genes tested in this study do not provide a useful biomarker of short term exposure to the tested doses of TH synthesis inhibiting pesticides.

Predictive Modeling of Thyroid Hormone Disruption and Investigation of Potential Biomarkers of Chemical Exposure

by

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DEDICATION

This work is dedicated to
my Gramp, Ned Flippin (1915-2006).

BIOGRAPHY

Jennifer Lynn Flippin is originally from Fort Seybert, West Virginia. Fort Seybert is a spot on the map in a large county in West Virginia that has very few people, one stoplight, and a newspaper that fits the entire county's weekly news into ten pages or less. While growing up, she spent many hours playing outside and gaining an appreciation of nature and the environment.

Flippin graduated first in her class from Pendleton County High School in 2001. She attended West Virginia University in Morgantown, WV where she received a Bachelor of Science degree in Biology and completed an undergraduate thesis under the direction of Dr. Christy Foran. Her studies examined the effect of a pharmaceutical product found in waste water on the reproduction of fish; her work was subsequently published in *Aquatic Toxicology*. Flippin graduated as one of the top 30 seniors at WVU in May 2005.

In August 2005 Flippin began graduate school at North Carolina State University in Raleigh, NC in the Department of Environmental and Molecular Toxicology. Her master's thesis focused on thyroid hormone disruption by chemical mixtures and was completed under the direction of Dr. Mike DeVito, and Dr. Kevin Crofton of the United States Environmental Protection Agency in Research Triangle Park, NC.

In the future, Flippin plans to become involved in field work, environmental education programs, and pursue a weekend career in photography.

> Your assumptions are your windows on the world. Scrub them off every once in a while or the light won't come in. If you challenge your own, you won't be so quick to accept the unchallenged assumptions of others. You'll be a lot less likely to be caught up in bias or prejudice or be influenced by people who ask you to hand over your brains, your soul, or your money because they have everything all figured out for you.
>
> --Alan Alda

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CHAPTER I.

INTRODUCTION

1. Endocrine disruptors in the environment

Environmental chemicals are capable of interacting and interfering with the endocrine systems of humans, wildlife, and aquatic organisms (Crisp et al, 1998; Gross-Sorokin et al 2006). An endocrine disruptor is "an exogenous agent that interferes with the production, release, transport, metabolism, binding, action, or elimination of natural hormones in the body responsible for the maintenance of homeostasis and the regulation of developmental processes" (Kavlock et al, 1996). Environmental chemicals interact with many components of the endocrine system, and may result in major regulatory disturbances of general physiology and development along with specific effects on the immune, reproductive, and nervous systems (Crisp et al, 1998).

Environmental contaminants may exhibit estrogenic, antiestrogenic, androgenic, antiandrogenic, and antithyroid activities (Brucker-Davis, 1998; Guillette and Gunderson, 2001; Boas et al., 2006). Pulp and paper mill effluents contain chemicals that act as androgens (Parks et al, 2001). Plastic additives like bisphenol A, and pesticides such as DDT act as estrogens in fish and wildlife (Guillette et al, 1994). Thyroid disrupting compounds occur naturally in food plants such as soy and members of the cabbage family (Doerge and Chang, 2002), as pharmaceuticals including methimazole (MMI) and propylthiouracil (PTU) (Ferreira et al, 2005; He et al., 2004), and as environmental pollutants (Brucker-Davis, 1998).

2. Overview of Thyroid Hormone Synthesis, Metabolism, and Regulation

2.1 Thyroid Hormone Production

In order to understand thyroid hormone disruption, it is important to recognize the normal function and purpose of thyroid hormones and their regulation. The thyroid gland is the endocrine organ responsible for the manufacture and release of two thyroid hormones, thyroxine (tetraiodothyronine,T_4) and triiodothyronine (T_3) (Figure 1). About 75% of the released product is T_4 while the remaining is T_3. Thyroid hormones function in developing, juvenile, and adult animals to regulate development, metabolism, and growth (Yen, 2001).

Production of T_3 and T_4 involves the conjugation of tyrosine residues and iodine within the thyroid gland (Connors, 1997; Zoeller et al, 2007) (Figure 2). Iodine is obtained via dietary intake, absorbed in the gastrointestinal tract, and transported to the thyroid by the circulatory system. Iodine is actively transported across the basal plasma membrane via the sodium-iodide symporter (NIS) where the thyroid can store and concentrate it at levels 20-40 fold over those found in circulation (Wolff, 1998). A Na+/K+ ATPase pump drives the NIS.

Iodine molecules are coupled with tyrosine residues supplied by large thyroglobulin (Tg) proteins (Cavalieri, 1997; Stevens, 1997). Thyroid peroxidase (TPO) in the presence of H_2O_2 catalyzes both the iodination of these tyrosine residues to form monoiodinated tyrosines (MIT) and diiodinated tyrosines (DIT) and also couples these iodinated products to form thyroid hormones (Taurog et al, 1996). Coupling two DIT molecules results in the formation of T_4; joining one DIT and one MIT forms T_3.

Hydrolases and proteases cleave T_3 and T_4 from thyroglobulin, and the hormones are released from the thyroid by passive diffusion.

2.2 Serum Transport of Thyroid Hormones

After release from the gland, T_3 and T_4 are bound to serum carrier proteins. In humans, approximately 75% of T_4 binds with high affinity to thyroxine binding globulin (TBG); the remaining 25% binds with transthyretin (TTR) and albumin (Schussler, 2000). In rats, binding occurs primarily with transthyretin. The different carrier proteins result in significant differences in serum half-lives for these hormones; the half-life of T_4 is 12-24 hours in rats and 5-9 days in humans (Döhler et al, 1979).

The active hormone that binds to nuclear receptors is T_3. Thyroxine (T_4) is considered a prohormone and must undergo a deiodination reaction to become T_3. This process takes place in a number of peripheral tissues (e.g., liver, brain) and is carried out by a family of enzymes known as deiodinases (Köhrle, 2007). Type I and Type II deiodinases are both responsible for the removal of one iodine molecule from T_4. Type I is expressed in the liver, kidney, and thyroid while Type II is mainly expressed in the brain.

2.3 Elimination of Thyroid Hormone by the Liver

The liver is the major organ that mediates elimination of xenobiotics and endogenous chemicals. Compounds undergo up to three phases of metabolism prior to excretion (Parkinson, 2001; Rose and Hodgson, 2004; LeBlanc and Dauterman, 2001). Phase I is carried out by enzymes like cytochrome P450s and functions to introduce a

polar group to a chemical, making it less reactive and more favorable for further metabolism. Phase II metabolism involves the addition of a bulky molecule (sugar, sulfate, amino acid, etc) to make a compound more water soluble and easily excreted in bile. Following metabolism, conjugated compounds excreted in bile are transported to the digestive tract where they are eliminated with other waste. Phase III elimination makes use of transporter proteins that facilitate the transfer of polar compounds across cell membranes.

Phase II metabolism and specifically the action of uridine diphosphate-glucuronosyl transferases (UGT) and sulfotransferases (SULT) catabolize T_3 and T_4 (MacKenzine et al., 2006; Kauffman, 2004). The metabolic mechanism in the rat involves UGT conjugation of T_4 with uridine 5'-diphosphoglucuronic acid (UDPGA); human T_4 metabolism is primarily mediated by SULTs and conjugation with 3'-phosphoadenosine 5'-phosphosulfate (PAPS) (You, 2004). UGTs are split into two families—UGT1 and UGT2 (MacKenzine et al., 2006). Members of each subfamily glucuronidate a broad spectrum of substrates, but not all isoforms metabolize the same compounds. The UGT1A1 and 1A6 variants are thought to be the specific enzymes responsible for T_4 clearance in the rat (Vansell and Klaassen, 2002). Phase III elimination including action by MCT8, MRP, MDR, and OATP also aid in the clearance of exogenous compounds and hormones from the body (Kretschmer and Baldwin, 2005). The OATP and MCT8 transporters are likely responsible for transport of THs into brain, and into and out of hepatocytes (Friesma et al., 2005; Hagenbuch, 2007).

2.4. Regulation of Thyroid Hormones

Normal regulation of thyroid hormones is a complex process that involves multiple tissues and is driven by negative feedback systems (Figure 3). The hypothalamic-pituitary-thyroid axis (HPT) regulates production of thyroid hormones. The hypothalamus plays a key role in integrating the endocrine and nervous systems; this portion of the brain receives information about hormone levels and secretes thyrotropin-releasing hormone (TRH) in response to decreased THs in circulation. Thyrotropin-releasing hormone is released into the adjacent anterior pituitary and stimulates production of thyroid stimulating hormone (TSH) which is released into the blood (Larsen et al, 2003; Zoeller et al, 2007).

Circulating TSH stimulates production of T_3 and T_4 in the thyroid gland. Receptors on the surface of thyroid follicle cells are bound by TSH, and through a cascade of cell signaling events, production of THs begin (Shupnik et al, 1989). Increased levels of THs in circulation produce a negative feedback on the hypothalamus and pituitary gland to inhibit T_4 production and maintain stable levels of THs. Both T_3 and T_4 directly depress activity of the TRH neurons in the hypothalamus and TSH release from the pituitary gland; thus stimulation of the thyroid gland decreases and production of THs is downregulated. As THs are cleared from circulation via metabolism by the liver and TH levels decrease, the negative feedback is removed and the hypothalamus and pituitary again release signals for hormone production (Larsen et al, 2003).

3. Thyroid Hormone Disruption

3.1 Thyroidal Hormone Disruptors

Since multiple tissues and mechanisms are involved in thyroid hormone synthesis, bioactivation, and elimination, it is not surprising that a variety of chemicals perturb different components of the HPT (Table 1). Some compounds directly inhibit TH production within the thyroid gland (Divi et al, 1997; Wolff, 1998). Iodine uptake is blocked by perchlorate, thiocyanate, and nitrates thus causing a decreased production of T_4 and T_3 (de Groef et al, 2006). The activity of TPO is inhibited by compounds including propylthiourea (PTU), methimazole, some pesticides, genistein found in soy products, and benzophenone 2, a UV light inhibitor (Schmulter et al, 2007; Capen 2001, Doerge and Chang, 2002**)**.

3.2 Extrathryoidal Hormone Disruptors

Binding of xenobiotics to the pregnane X receptor (PXR), the constitutive androstane receptors (CAR), and the aryl hydrocarbon receptor (AhR) regulate the expression of phase I, II, and III proteins in the liver (Kretschmer and Baldwin, 2005; Tompkins and Wallace, 2007). Activation of CAR, PXR, and AhR induce UGTs and SULTs (Figure 4), phase II enzymes which catabolize T_3 and T_4 (Shelby and Klaassen, 2006; Qatanani et al, 2005; Beestra et al, 1991). Polychlorinated dibenzo-*p*-dioxin (PCDD), polychlorinated dibenzofurans (PCDFs), and some PCBs are activators of AhR (Van den Berg et al, 2006). Those PCBs that activate AhR are considered “dioxin-like” and include PCBs-77, 126, and 169. Many other PCBs are described as “phenobarbital-like” because of their ability to bind with rodent PXR or CAR, induce CYP2B along

with UGTs (Czekj, 2000). Congeners shown to activate this pathway in rodents include PCBs-153, 184, and 197 (Tabb et al., 2004).

Other extrathyroidal locations where hormone disruption occurs include deiodinases and serum binding proteins. Peripheral tissue deiodinases are inhibited by xenobiotics including the food dye FD&C Red # 3. This inhibition causes decreased monodeiodination of T_4, and leads to reduced serum T_3 and increased serum T_4 (Capen, 1997). Binding of THs to serum binding proteins like transthyretin can be inhibited by hydroxy-PCBs and other chemicals (Lans et al, 1993).

3.4 Implications of Thyroid Hormone Disruption

Thyroid hormones are import in both adult and developing organisms. In adults, THs are important for regulation of metabolism, but they are also key transcriptional activators of in bone, heart, fat, liver, pituitary, and brain (Yen, 2001). During development THs play a critical role in somatic growth. Low circulating levels of THs during development in animals have been linked with neurological problems and structural brain defects (Zoeller and Crofton, 2005; Koibuchi and Chin, 2000). Maternal TH levels may play a vital role in early brain development of offspring. Thyroid hormones are important from the time of mature oocyte formation and throughout the rest of gestation (Howdeshell, 2002). Human *in utero* exposure to thyroid disrupting chemicals may be linked with neurodevelopmental and behavioral problems including attention deficit hyperactivity disorder and autism (Colborn, 2004). It is clear that thyroid hormones play a critical role in both developing and adult organisms, and that alterations in thyroid hormone concentrations are detrimental.

4. Description of Two Classes of Thyroid Disrupting Chemicals

4.1 Polyhalogeneated Aromatic Hyrdrocarbons

Polyhalogenated aromatic hydrocarbons (PHAHs) are a class of persistent, bioaccumulative environmental chemicals that include polychlorinated biphenyls (PCBs), dioxins, and furans (Figure 5). Such chemicals are found far from their original sources and are ubiquitous in the environment due to biotic and abiotic transport. They are problematic due to their persistence, bioaccumulation, and toxicity. Exposure results from consumption of contaminated food products, but also may occur due to accidental or occupational exposure (Domingo, 2007; Travis and Hattemer-Frey, 1991).

Polychlorinated biphenyls (PCBs), once valued for their nonflammable and insulating properties, were used as coolants and lubricants in transformers and electrical equipment (ATSDR, 2001). They were manufactured as solids or "oily liquids," but production in the United States ceased in 1977 due to their environmental persistence and evidence of adverse health effects. Dioxins and furans have never been produced for specific commercial use; rather they are an unintended byproduct of industrial processes. Sources have historically included pulp and paper mills, incinerators or other combustion sources, and chemical manufacture (Fiedler, 1996). The chlorophenoxy herbicide Agent Orange, a combination of 2,4-D and 2,4,5-T is an example of a product contaminated by 2,3,7,8 tetrachlorodibenzo-p-dioxin (TCDD) during production.

Dioxins, furans, and PCBs are agonists of CAR, PXR, and AhR (Kretschmer and Baldwin, 2005). Dioxin-like compounds including TCDD activate AhR (Van den Berg et al, 2006) while nondioxin-like compounds such as PCB-153 are activators of rodent

PXR or CAR (Tabb et al., 2004). Activation of these hepatic nuclear receptors increases expression of UGTs and SULTs that catabolize T_3 and T_4 (Vansell and Klaassen, 2002).

4.2 Pesticides: Pronamide, Mancozeb, and Thiram

Pronamide is a selective herbicide related to the organochlorine class of chemicals that is used to control certain broadleaf weeds and grasses (Figure 6). It is a pre- or post- emergence herbicide that acts to inhibit plant cell division. It is primarily applied to lettuce, blueberries, fruit trees, ornamental trees and shrubs, turf, and some species of legumes (Extension Toxicology Network, 1996). Pronamide is persistent in soil and resists hydrolysis and photolysis. It is stable for greater than 13 months under anaerobic conditions, and leaching may introduce the chemical to water systems (EPA, 1994). The main target organs for toxicity are the liver, thyroid gland, testes, and pituitary gland.

Mancozeb is a dithiocarbamate fungicide (Figure 5) used to protect fruit, vegetable, grain, turf, flower, and nut crops and stored seeds from fungal diseases (fruit scabs, blights, leaf spot, etc) (Extension Toxicology Network, 1996). Approximately 5.6 million pounds are applied annually with the greatest amount used on apples, potatoes, and grapes (EPA, 2006). Thiram is also a dithiocarbamate fungicide and is used on a variety of fruits, vegetables, ornamental species, and turf crops. Additionally, its use is extended as a seed protectant, animal repellent, and was previously used for treatment or prevention of certain human skin conditions including scabies, bacterial infection, and sun burn (Extension Toxicology Network, 1996). Over 165,000 pounds of thiram are

applied to 35,000 acres of strawberries, apples, and peaches every year and another 631,000 pounds are used to treat approximately 1.3 billion pounds of seeds (EPA, 2004).

Human exposure to all of these pesticides occurs through consumption of contaminated food products, water, or accidental exposures. Dietary exposure occurs when residual chemicals remain on food products and are ingested. The major routes of exposure to pesticide handlers, workers, and homeowners is via dermal and inhalation routes (EPA, 2002).

In addition to the herbicidal and fungicidal effects of pronamide, mancozeb, and thiram, these chemicals are also thyroid hormone disruptors. Short term *in vivo* exposures in laboratory animals show mancozeb, thiram, and pronamide reduced serum concentrations of T_4 and increased thyroid mass (Raizada RB et al., 1979; Nebbia et al., 1995; Nebbia and Fink-Gremmels, 1996; Kackar et al., 1997; Ivanova-Chemishanska et al., 1971; Hedge et al abstracts). The primary mode of action of these pesticides is thought to be through TH synthesis inhibition and possibly by TPO inhibition. Chemicals that are TPO inhibitors also decrease circulating T_3; however, these effects occur at higher exposures than those that begin to decrease T_4. Increased concentrations of circulating TSH in humans has been observed in individuals who used fungicides in an occupational setting (Panganiban et al., 2004; Steenland et al., 1997)

The mechanism of action of thiram and pronamide are not completely understood but are likely similar to other TPO inhibitors. Chinese hamster ovary cells transfected with the human TPO gene show no effect of thiram on TPO inhibition when using iodination of a Glu-Tyr-Glu peptide and oxidation of guiacol as endpoints (Marinovich et al, 1997). However, TPO is complex enzyme that functions beyond iodination, such

as the coupling of monoiodinated tyrosine (MIT)/ diiodinated tyrosine (DIT) to make T_3 and T_4 (Larsen et al, 2004; Taurog et al., 1996). Thus while thiram may not inhibit iodination or oxidation of guiacol *in vitro*, it may inhibit those processes *in vivo* or inhibit other functions of TPO involved in TH synthesis. Other data indicate more similarities between thiram and pronamide with other TPO inhibitors than thyroid hormone disruptors that induce UGTs or inhibit deiodination. Exposure to thiram and pronamide results in significant decreases in T_4 in rats in a pattern similar to other TPO inhibitors such as mancozeb (Hurley et al., 1998; Hedge et al., 2002). In these studies, there was no induction of T_4-glucuronidiation or enzymatic markers of CYP induction (EROD and PROD). Thus thiram and pronamide are not likely to decrease THs through hepatic effects. Additionally, they are not likely to be specific inhibitors of deiodinases because exposure does not cause a significant increase in circulating T_4, a hallmark of FD&C Red No. 3, a specific deiodinase inhibitor (Capen 1997). Therefore, for the purpose of this study, thiram and pronamide were placed into the TH synthesis inhibitor group with mancozeb, a known inhibitor of TH synthesis (Hurley et al, 1998).

5. Predictive Modeling

Complex mixtures of xenobiotics including PHAHs, pesticides, pharmaceuticals, and metals are evident in water (Kolpin et al, 2004), food sources (Riedel et al, 2002; Domingo and Bocio, 2007), and human tissue (Turyk et al., 2006). Individual components of chemical mixtures are not often found in high concentrations, but previous research demonstrates that chemicals at low doses can act in a dose additive manner to affect an endocrine endpoint (Crofton et al, 2005).

In general, two approaches are used to make predictions about the toxicity of a chemical mixture—dose addition and effect addition. Dose addition can be used to predict the effects of chemicals that elicit a toxic response through the same mechanism of action (Drescher and Boedeker, 1995). For example, all chemicals that upregulate the expression of UGTs and cause an increased clearance of T_4 can be said to be acting through a similar mechanism of action. Dose addition can be described mathematically by Equation 1:

$$R = \frac{1}{1 + \frac{1}{\left(\sum_{i=1}^{n} \frac{D_i}{ED50_i}\right)^{\rho'}}}$$

Equation 1

where R is the response to the mixture, D_i is the dose, ED_{50i} is the dose of chemicals that results in a 50% decrease in T_4, and ρ' is the average slope factor of all chemicals grouped with the same mode of action.

Effect addition describes mixture toxicity of chemicals that act through different mechanisms of action and assume no interactions of the effects of different mechanisms. For example, the responses to a chemical acting as a TH synthesis inhibitor and a chemical acting as a UGT inducer can be summed via effect addition to predict their effect on circulating T_4 concentrations. Effect addition can be described mathematically by Equation 2:

$$R = 1 - \prod_{i=1}^{n} (1 - R_i)$$

Equation 2

where R is the response to the mixture, and R_i represents the response to chemical in cassette *i*.

Neither dose addition nor effect addition alone is adequate to describe the effects of most environmentally-relevant mixtures; many consist of chemicals that act through similar and different mechanisms of action. Olmstead and LeBlanc (2005) developed a model combining dose addition with effect addition to accurately predict toxicity of a mixture with similar and dissimilar components. In the present study, the contribution of PHAHs was calculated by dose addition, based on a similar mechanism: upregulation of UGTs and enhanced hepatic clearance of T_4 (Vansell and Klaassen, 2002). Additionally the effect of the pesticides was calculated as a second cassette based on a shared mechanism: decreased hormone synthesis (Doerge and Takazawa, 1990). The total response to the mixture with PHAHs and pesticides was calculated using effect addition (Figure 7). Thus the final model output described the decrease in T_4 due to both upregulation of hepatic catabolism and inhibition of TH production.

The integrated addition model is described mathematically in Equation 3.

$$R = 1 - \prod_{I=1}^{N} \left\{ 1 - \frac{1}{1 + \frac{1}{\left(\sum_{i=1}^{n} \frac{D_i}{ED50_i} \right)^{\rho_{avg,I}}}} \right\}$$

Equation 3

6. Thyroid Gene Expression as a Biomarker of Chemical Exposure

Gene expression patterns in the thyroid may serve as useful biomarkers to determine endocrine disruption and pathological endpoints (Glatt et al., 2005). Transcription of RNA is an important step in upregulation and downregulation of thyroid activity. Some of the most important genes involved in this process are transcription factors and genes that directly regulate protein synthesis. These include the haematopoietically expressed homeobox (HEX), paired box gene 8 (PAX-8), thyroid transcription factor 1 and 2 (TTF-1, TTF-2), thyroid hormone receptor (TSHr), sodium iodine symporter (NIS), thyroglobulin (Tg), thyroid peroxidase (TPO) and dual oxidase 1 and 2 (DUOX 1, DUOX 2) (Lacroix et al, 2006).

The relationship among thyroidal genes is complex and not yet completely understood, but their expression seems to be partially dependent on one another. Following direct interaction of PAX-8 and TTF-1 the complex binds to the promoter regions of TPO, Tg, and TSHr genes to result in increased transcription (Di Palma et al, 2003; Lacroix et al, 2006). Thyroglobulin provides negative feedback on TTR-1 and PAX-8 and also suppresses the mRNA levels of NIS, TPO, Tg, and the TSH receptor (Suzuki et al, 1998; Suzuki et al, 1999). Hex has been reported to be a repressor of the Tg promoter and thus attenuates the activating effects of TTF-1 and PAX-8 to stimulate transcription (Martinez et al, 2000; Pellizzari et al, 2000).

Evidence suggests that chemicals that inhibit TPO lead to an increase in transcription of certain mRNA. Thyroid cells exposed to methimazole (MMI) and propylthiouracil (PTU), antithyroid drugs that inhibit TPO, increase expression of TPO (Sugawara et al., 1999; Isozaki et al., 1991) and Tg (Leer et al., 1991) in a TSH-

independent manner. Thus, in addition to reducing T_4 production, TPO inhibitors alter gene expression in the thyroid independent of changes in TSH.

7. Hypotheses and Specific Aims

7.1 Hypothesis and Specific Aim I

Hypothesis I: Decreased serum T_4 concentration in rats exposed to a mixture of TH synthesis inhibitors and hepatic enzyme inducers is best predicted by an integrated addition model.

Specific Aim I: Rats were exposed to dilutions of a mixture of PHAHs and TH synthesis inhibitors that respectively increase clearance or decrease production of THs. Serum T_4 was measured following exposure. Empirical results of T_4 concentrations were compared with predictions generated by three models—dose addition, effect addition, and integrated addition. Models were compared to determine how far empirical data deviated from predictions.

7.2 Hypothesis and Specific Aim II

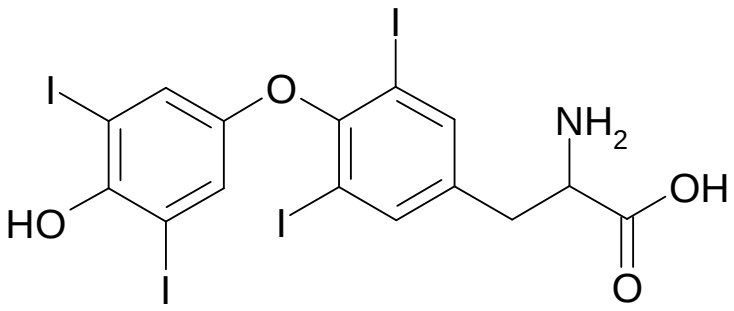
Hypothesis II: Thyroidal mRNA levels of PAX-8, Hex, Tg, NIS, and/or TPO can be utilized as biomarkers to indicate animal exposure to chemicals that are TH synthesis inhibitors.

Specific Aim II: Genes that code for thyroid transcription factors and proteins utilized in TH synthesis show altered expression when exposed to the TH synthesis inhibitors, propylthiouracil or methimazole. Most of these effects have been shown *in vitro* and little information is available on *in vivo* thyroid gene transcription following

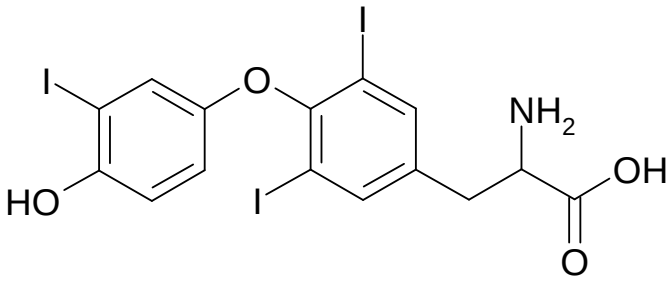
short term exposure to chemicals that interact with the thyroid gland. Rats were dosed with TH synthesis inhibitors and rtPCR was used to determine if gene expression changed in response to exposure.

8. Significance of Work

This research helps to present a clearer understanding of how T_4 levels are affected when TH synthesis is inhibited and hepatic microsomal enzymes are induced by chemical mixtures. Additionally, preliminary studies show that certain gene expression patterns in the thyroid gland are not useful to determine if exposure to TH synthesis inhibitors has occurred. The results of this study contribute to the knowledge of the effects of mixtures on circulating thyroid hormone concentrations and the ability of mathematical models to predict this endpoint. Humans and wildlife are not merely exposed to single chemicals in their environments so it is not realistic to assume that a single chemical is acting alone to produce an endocrine disruption event. Additionally, the study provides insight that while some thyroidal genes result in upregulation following exposure to TH synthesis inhibitors, these responses are not sensitive indicators of low level thyroid hormone disruption. Future studies will likely continue to examine the effects of mixtures and also develop biomarkers of exposure to components of these mixtures.



T_4



T_3

Figure 1. Structure of thyroid hormones: thyroxine (T_4) and triiodothyronine (T_3).

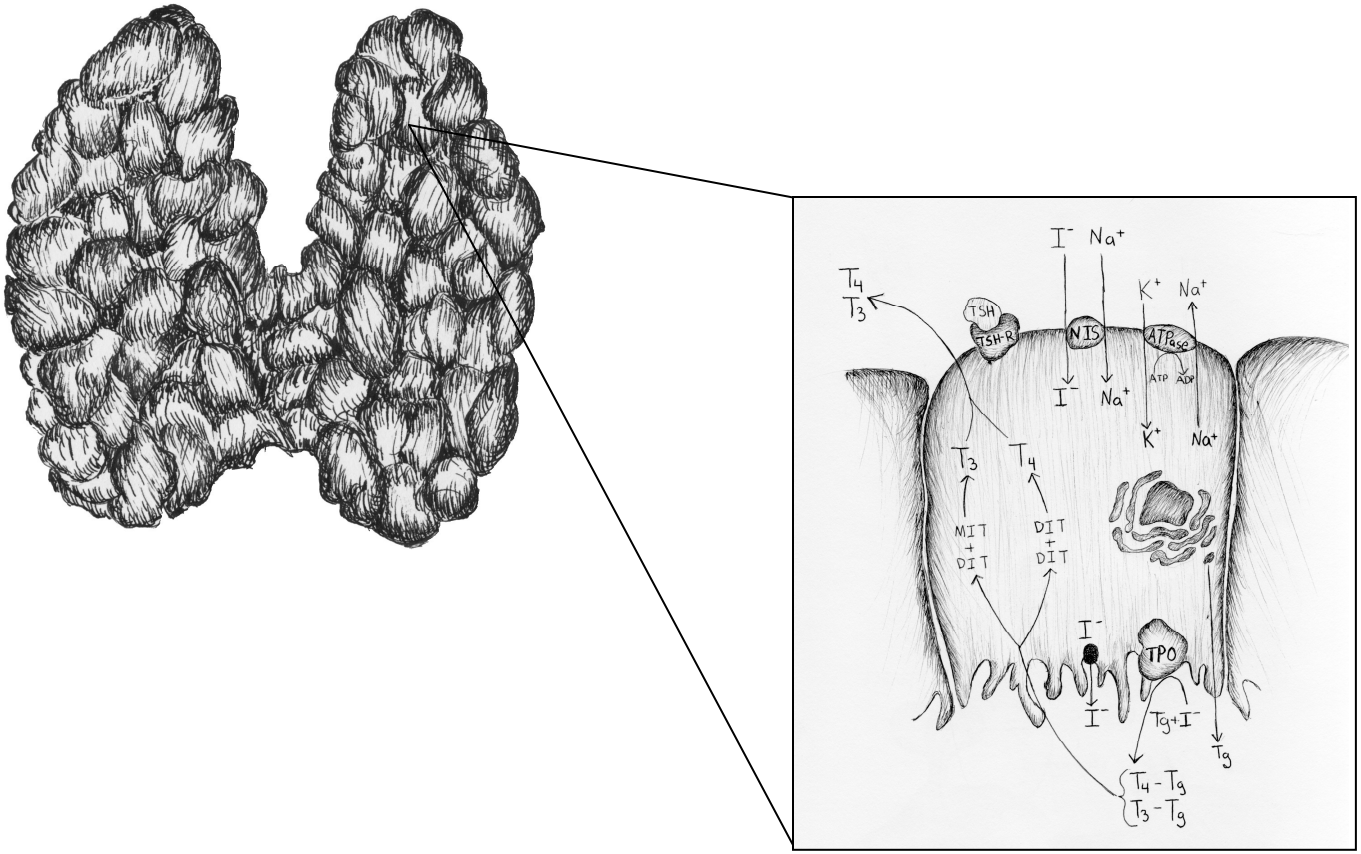


Figure 2. Production of thyroid hormones within the thyroid gland. Iodine (I) enters thyroid follicle cells via the sodium iodide symporter (NIS). The thyroid peroxidase enzyme (TPO) combines I^- with a tyrosine residue on a thyroglobulin (Tg) protein and also couples the iodinated residues for hormone synthesis. If two diiodinated tyrosines (DIT) are combined, T_4 results; if a monoiodinated tyrosine (MIT) and a DIT are combined, T_3 results. Hormones are released into circulation via passive diffusion. Illustration by J.L. Flippin; modified from Larsen et al, 2004.

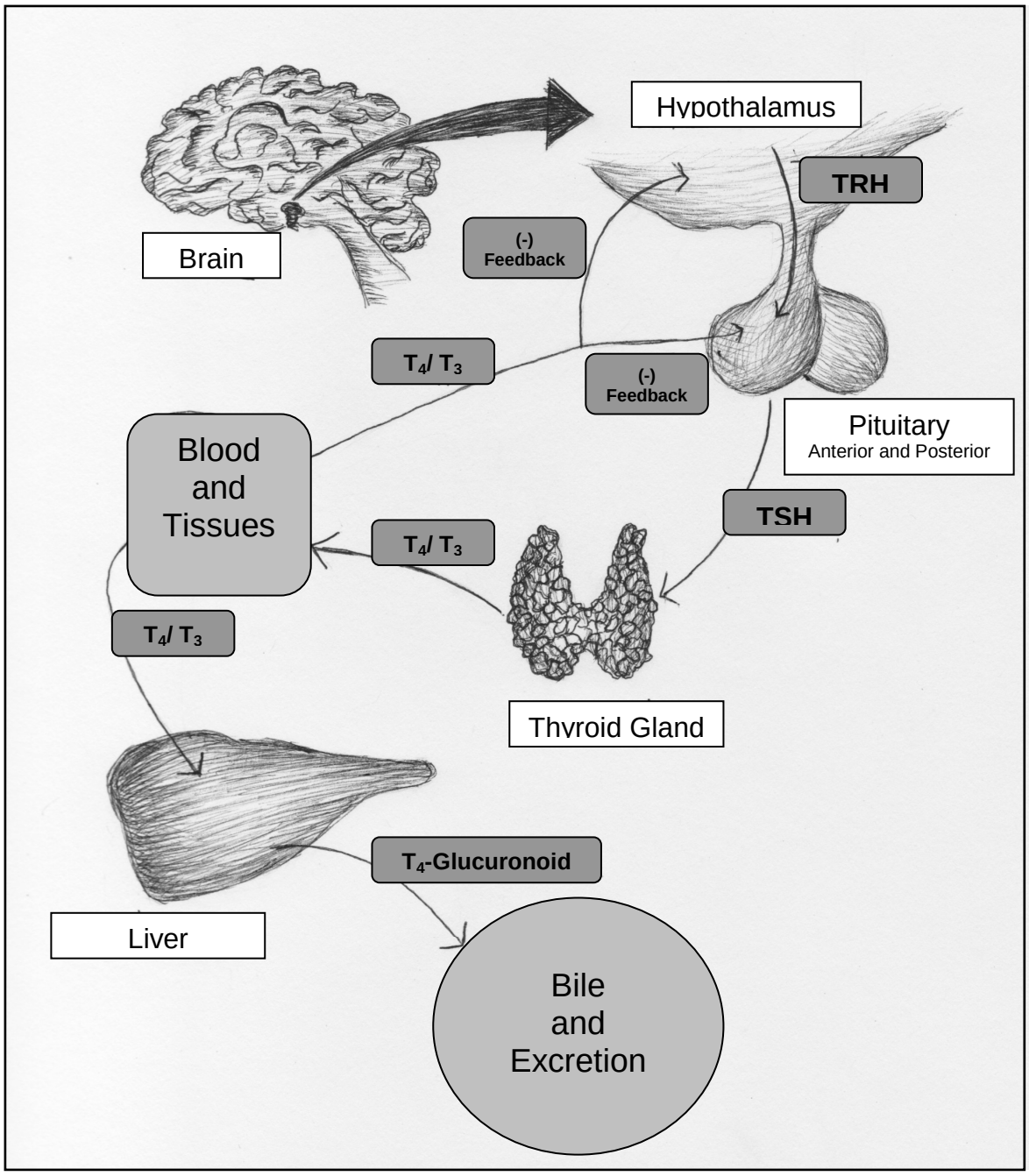
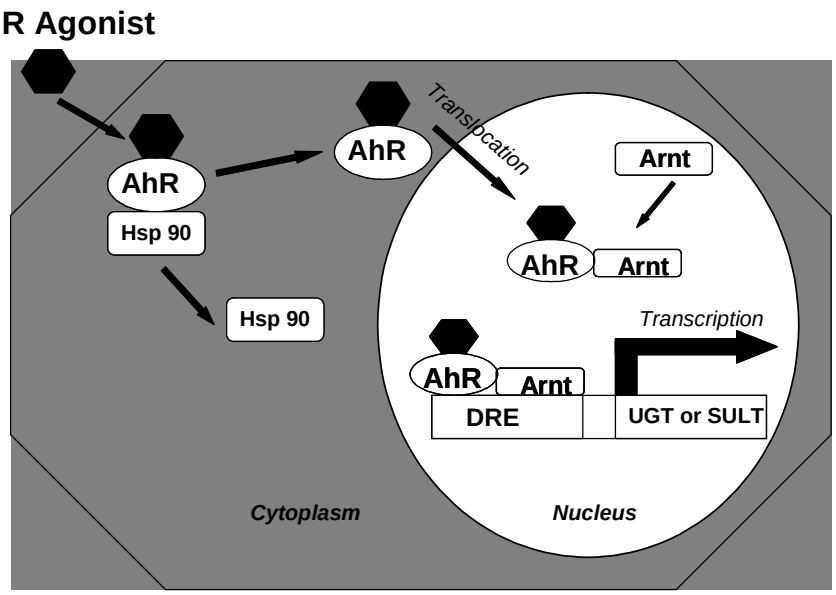


Figure 3. Thyroid hormone production, metabolism, and regulation by negative feedback. In response to low levels of circulating thyroid hormones (THs), the hypothalamus produces thyrotropin-releasing hormone (TRH) and releases it to the anterior pituitary gland which subsequently excretes thyroid stimulating hormone (TSH) into circulation. TSH binds to receptors on the thyroid gland, and stimulates production of T_4/T_3. Circulating hormones negatively feedback on the pituitary and hypothalamus and downregulate TH production. They are eventually catabolized by Phase II and III metabolism, and excreted in bile for removal from the body. Illustration by J.L. Flippin; modified from Larsen et al, 2004; Zoeller et al. 2007.

4a)



4b)

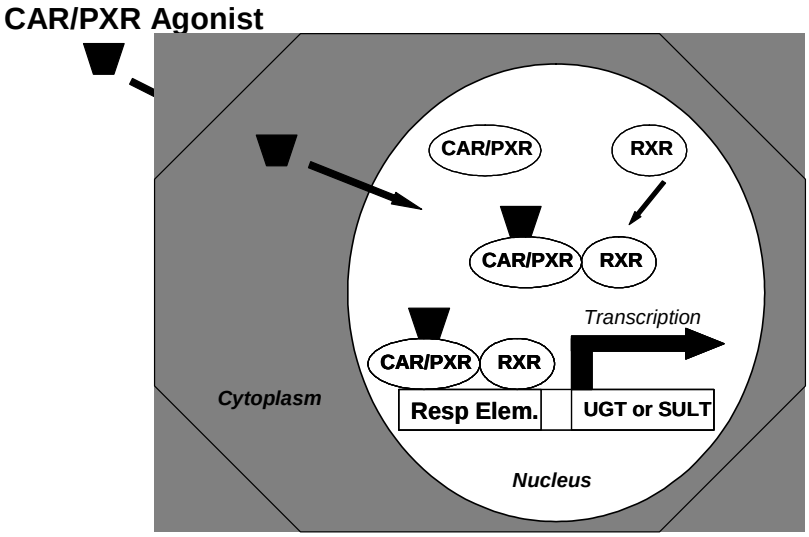
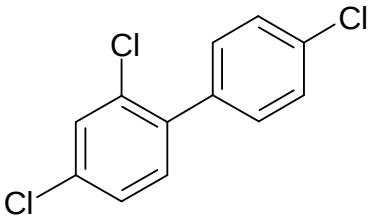
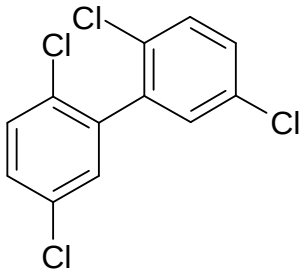


Figure 4. Activation of AhR and CAR/PXR and up regulation of UGTs or SULTs in hepatocytes. a) AhRs are complexed with Heat Shock Protein 90 (Hsp90) in the cytoplasm until activator binding. Bound AhR dissociates from Hsp90 and translocates to the nucleus where it binds to the Arnt protein. The complex binds with a DNA response element and stimulates transcription of UGTs or SULTs. AhR is activated by dioxin-like chemicals. b) CAR and PXR are found in the nucleus and both share a similar mechanism of action. CAR/PXR, bound by an activator, complexes with RXR. This complex binds with a DNA response element to stimulate transcription of UGTs or SULTs. Activators of CAR/PXR are phenobarbital-like chemicals. Modified from Tompkins and Wallace, 2007; Kretschmer and Baldwin, 2005; You, 2004.

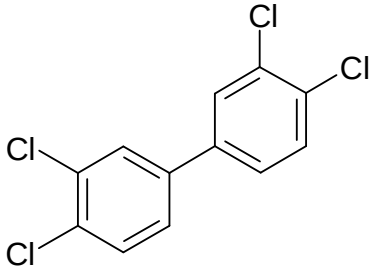
Figure 5. Polyhalogenated aromatic hydrocarbons (PHAHs) used in the mixture tested in this study. PHAHs consist of PCBs, dioxins, and furans. PCBs were once manufactured for industrial use; dioxins and furans occur as byproducts of manufacturing. These compounds are activators of AhR, PXR/CAR and lead to increased clearance of THs.



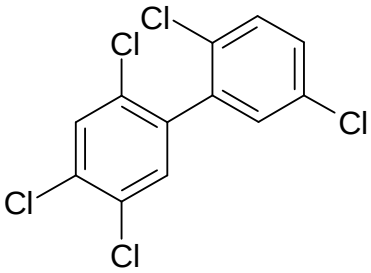
PCB 28



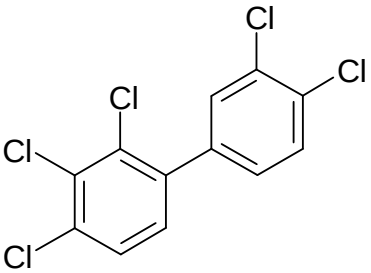
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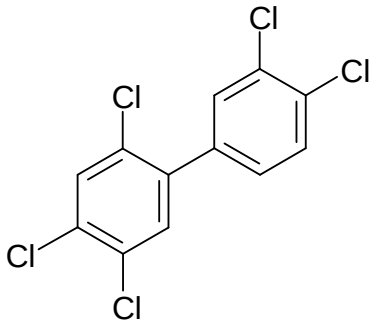
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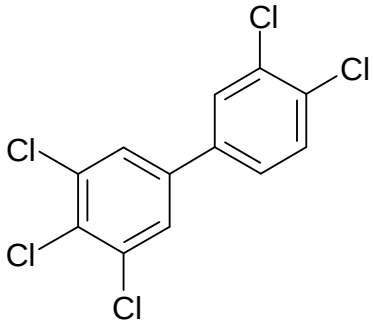
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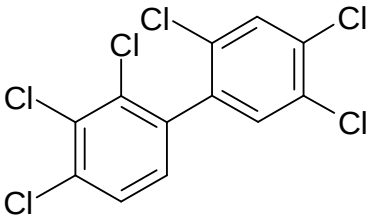
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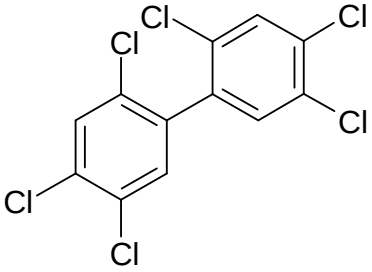
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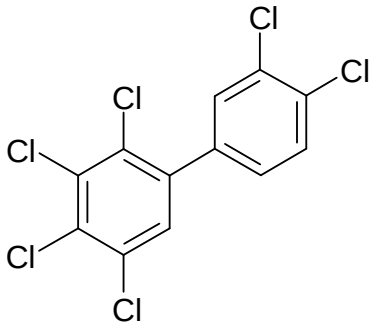
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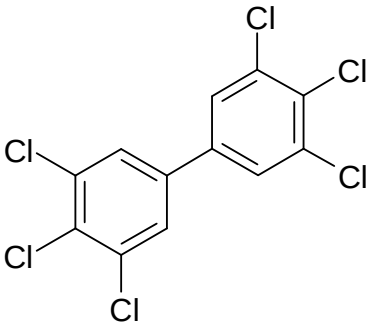
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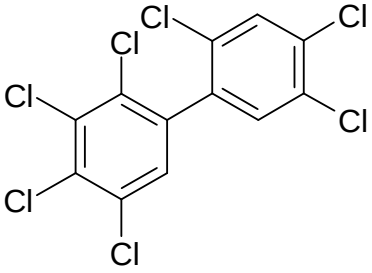
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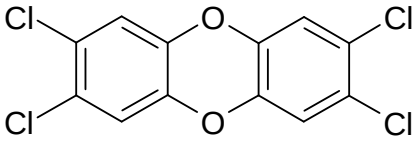
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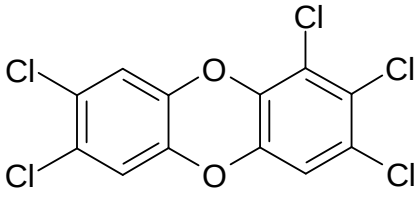
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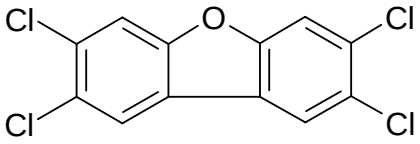
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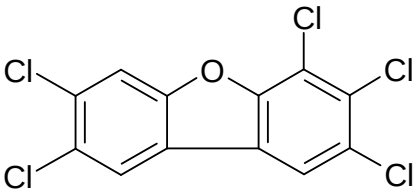
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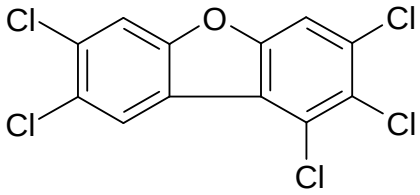
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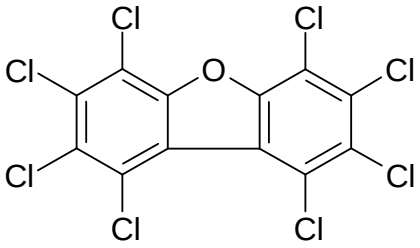
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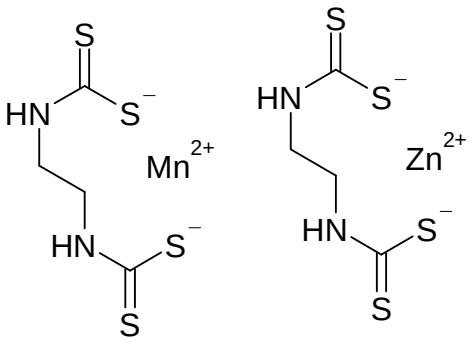
1-PCDF



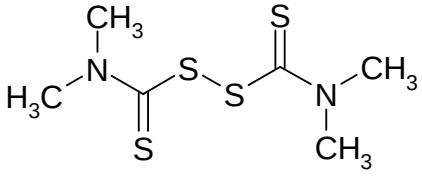
4-PCDF



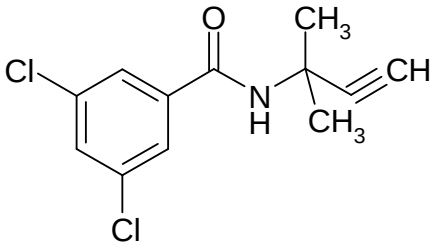
OCDF



Mancozeb



Thiram



Pronamide

Figure 6. Pesticides used in the mixture tested in this study. Mancozeb and thiram are fungicides and pronamide is an herbicide. These compounds are thyroidal TH synthesis inhibitors.

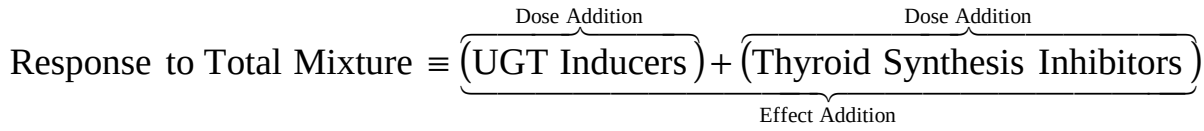


Figure 7. Schematic depiction of integrated addition model. Dose additivity is calculated within cassettes for chemicals that share the same mechanism of action; effect additivity is calculated between cassettes for chemicals with different modes of action.

Table 1. Classes, mechanisms of action, and effects of thyroid hormone disrupting compounds (modified from Crofton, unpublished)

Class	Mechanism	Effects on THs	Chemicals	References
Iodine Transport	Competition/blockage of NIS	Decreased thyroidal synthesis of T_3 and T_4	Perchlorate, chlorate, bromate	(Van Sande et al., 2003; Wolff, 1998)
Synthesis Inhibitors	Inhibition of TPO	Decreased thyroidal synthesis of T_3 and T_4	Methimazole, amitrole, mancozeb, soy isoflavones, benzophenone 2	(Capen 1997; Doerge and Sheehan, 2002; Hurley, 1998; Schmutzler, 2007)
Transport Disruption	Altered binding to serum transport protein	Unknown	Hydroxyl-PCBs, EMD 49209, pentachlorophenol	(Lans et al., 1993; Schroder-van der Elst et al., 1997; van den Berg, 1990)
Enhanced Hepatic Catabolism	Upregulation of glucuronylsyltransferases or sulfotransferases via CAR/PXR or AhR	Increased biliary elimination of T_3 and T_4	Acetochlor, Phenobarbital, 3-methylcolanthrene; PCBs	(Brucker-Davis, 1998; Hood and Klaassen 2000; Hurley, 1998)
Deiodinase Inhibitors	Inhibition of deiodinases	Decreased peripheral synthesis of T_3	FD&C Red Dye #3; propylthiouracil; octyl-methoxycinnamate	(Capen, 1998; Visser et al., 1979; Klammer et al., 2007)
Thyroid Receptor Agonists and Antagonists	Direct or indirect competition of TR-TRE binding	Decreased activation of TH dependent gene transcription	Tetrabromo Bisphenol A, Bisphenol A, Hydroxy PCBs	(Kitamura et al, 2005; Moriyama et al., 2002)

CHAPTER II

Predictive Modeling of a Mixture of Thyroid Hormone Disrupting Chemicals that Affect Production and Clearance of Thyroxine (T_4)

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Research Triangle Park, North Carolina

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Abstract

Humans are exposed to chemical mixtures via diet and other accidental exposures. Mixtures have previously been shown to exert additive effects on endocrine endpoints. Thyroid hormone disrupting compounds interfere with both thyroidal and extrathyroidal mechanisms to decrease circulating thyroxine (T_4). This research tested the hypothesis that serum T_4 concentrations of rodents exposed to a mixture of both TH synthesis inhibitors (pesticides) and stimulators of T_4 clearance in the liver (PHAHs) could be best predicted by a model of integrated addition. Female Long-Evans rats, 23 days of age, were dosed with dilutions of a mixture of 18 PHAHs (2 dioxins, 4 dibenzofurans, and 12 PCBs, including dioxin-like and non-dioxin like PCBs) and a mixture of 3 pesticides (thiram, pronamide, and mancozeb) for four consecutive days. Serum was collected 24 hours after the last exposure and T_4 concentrations were measured by RIA. Animals exposed to the highest dose of the mixture experienced a 45% decrease in serum T_4. Three additivity model predictions (dose addition, effect addition, and integrated addition) were generated based on single chemical data, and the results were compared. The response to this mixture was best modeled by integrated addition in which responses to the pesticides and PHAHs were separately evaluated according to dose additivity and the response to the two doses of chemicals were summed according to effect additivity. The response to the mixture was also well predicted by a dose addition model. These results lead to a clearer understanding of thyroid hormone disruption by a complex mixture and show that models are able to accurately predict the response of T_4 to a mixture.

Introduction

Environmental chemicals interact and interfere with the endocrine systems of humans, wildlife, and aquatic organisms (Crisp et al, 1998; Gross-Sorokin et al 2006). An endocrine disruptor is defined as "an exogenous agent that interferes with the production, release, transport, metabolism, binding, action, or elimination of natural hormones in the body responsible for the maintenance of homeostasis and the regulation of developmental processes" (Kavlock et al, 1996). Environmental contaminants exhibit estrogenic, antiestrogenic, androgenic, antiandrogenic and antithyroid activities (Guillette and Gunderson, 2001; Brucker-Davis, 1998)

Xenobiotics that disrupt thyroid hormone (TH) homeostasis act via effects at the thyroid gland as well as by extrathyroidal mechanisms (Boas et al., 2006, Brucker-Davis, 1998, DeVito et al., 1999). Suppression of TH production or secretion from the thyroid results from inhibition of iodine uptake, thyroid peroxidase activity, or TH secretion (de Groef et al, 2006; Capen, 1994). Increased clearance of THs can result from exposure to chemicals that upregulate TH metabolizing enzymes in the liver. Exposure to some food products (Doerge and Chang, 2002), pharmaceuticals (He et al., 2004; O'Connor et al., 1999), and environmental chemicals (Brucker-Davis, 1998) can alter synthesis or hepatic elimination of THs.

Chemicals decrease thyroidal synthesis of THs by several mechanisms. Perchlorate and thyocyanate decrease iodine uptake by inhibiting the sodium iodide symporter (NIS). Thyroid peroxidase (TPO) is inhibited by chemicals including propylthiouracil, methimazole, sulfonamides, and ethylthiourea, a metabolism product of

dithiocarbamate fungicides (McClain, 1995; Doerge and Takazawa, 1990). Lithium inhibits TH release from the thyroid (Capen, 1992).

In short term *in vivo* lab animal exposures, dithiocarbamate fungicides such as mancozeb and thiram, and the herbicide pronamide reduced serum concentrations of T_4 and increased thyroid mass (Raizada RB et al., 1979; Nebbia et al., 1995; Nebbia and Fink-Gremmels, 1996; Kackar et al., 1997; Ivanova-Chemishanska et al., 1971; Hedge et al, 2002). The primary mode of action of these pesticides is thought to be through TH synthesis inhibition. This is evidenced by decreases in TPO activity *in vitro* by mancozeb (Kackar et al., 1997). Thyroid hormone synthesis inhibitors also decrease circulating T_3; however, these effects occur at higher exposures than those that begin to decrease T_4. Increased circulating concentrations of thyroid stimulating hormone (TSH) are observed in individuals who used fungicides in an occupational setting (Panganiban et al, 2004; Steenland et al., 1997), but these hormone changes are not usually observed in four-day lab animal assays.

Extrathyroidal mechanisms of TH action include increases in the hepatic catabolism of T_4 resulting from activation of hepatic nuclear receptors. Binding of xenobiotics to the pregnane X receptor (PXR), the constitutive androstane receptor (CAR), and the aryl hydrocarbon receptor (AhR) can induce expression of phase I, II, and III metabolism proteins (Kretschmer and Baldwin, 2005; Tompkins and Wallace, 2007). Activation of CAR, PXR, and AhR induce UGTs and SULTs, phase II enzymes which catabolize T_3 and T_4 (Shelby and Klaassen, 2006; Qatanani et al, 2005; Beestra et al, 1991). Polychlorinated dibenzo-*p*-dioxin (PCDD), polychlorinated dibenzofurans (PCDFs), and some PCBs are activators of AhR (Van den Berg et al, 2006). Those PCBs

that activate AhR are considered “dioxin-like” and include PCBs-77, 126, and 169. Many other PCBs are described as “phenobarbital-like” because of their ability to bind with PXR or CAR and induce CYP2B along with phase II enzymes such as UGTs (Czekj, 2000). Congeners shown to activate this pathway in rodents include PCBs-153, 184, and 197 (Tabb et al., 2004).

Mixtures of PHAHs, pesticides and other chemicals are commonly found in food and environmental matrices (Riedel et al, 2002; Domingo and Bocio, 2007; Kolpin et al, 2004). Estimating the potential human health risks associated with such exposures requires the use of single chemical data along with mathematical models. Models for assessing the risk of mixtures often employ the concepts of dose and effect additivity to describe the effects of a complex mixture of chemicals affecting the same end point but acting through similar and/or dissimilar modes of action. Dose addition describes the behavior of chemicals with the same mechanism of action and assumes that the sum effect of the mixture is based on the potency of each compound (US EPA, 2000). Effect addition is used to estimate the effects of a mixture of chemicals with different mechanisms of action (US EPA, 2000). Some models are designed to integrate both the concepts of dose addition and effect addition in order to address complex mixtures of chemicals with both similar and dissimilar modes of action. Olmstead and LeBlanc (2005) describe a model that calculates dose addition among chemicals suspected to have the same mechanisms of action and effect addition between groups of chemicals with different mechanisms of action. We tested the hypothesis that decreased serum T_4 concentration in rats exposed to a mixture of TH synthesis inhibitors and hepatic enzyme

inducers is best predicted by an integrated addition model as compared to either a dose addition or an effect addition model.

Materials and Methods

Chemicals: Three pesticides were purchased from ChemService (West Chester, PA): thiram (CAS# 137-26-8; catalog# PS-24; purity: 98%), pronamide (CAS# 23950-58-5, catalog# PS-349; purity: 98.3%), and mancozeb (CAS# 8018-01-7, catalog# PS-2095, purity: technical grade). Dilutions for each individual pesticide were made in corn oil (Sigma Aldrich) to result in the following doses: thiram (6.25, 12.5, 25.0, 50.0, 100, 200, 400, and 800 mg/kg); mancozeb (3.90, 7.80, 15.6, 31.3, 62.5, 125, 250, 500, and 1000 mg/kg); pronamide (3.90, 7.80, 15.6, 31.3, 62.5, 125, 250, 500, and 1000 mg/kg).

A custom mixture of 18 PHAHs in a corn oil vehicle was prepared by Cambridge Isotope Laboratories for Crofton et al (2005). The mixture consisted of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD); 1,2,3,7,8-pentachlorodibenzo-p-dioxin (PCDD); 2,3,7,8 tetrachlorodibenzofuran (TCDF); 1,2,3,7,8-pentachlorodibenzofuran (1-PCDF); 2,3,4,7,8-pentachlorodibenzofuran (4-PCDF); 1,2,3,4,6,7,8,9-octachlorodibenzofuran (OCDF); 2,4,4´-trichlorobiphenyl (PCB-28); 2,2´,5,5´-tetrachlorobiphenyl (PCB-52); 3,3´,4,4´-tetrachlorobiphenyl (PCB-77); 2,2´,4,5,5´-pentachlorobiphenyl (PCB-101); 2,3,3´,4,4´-pentachlorobiphenyl (PCB-105); 2,3´,4,4´,5-pentachlorobiphenyl (PCB-118); 3,3´,4,4´,5-pentachlorobiphenyl (PCB-126); 2,2´,3,4,4´,5´-hexachlorobiphenyl (PCB 138); 2,2´,4,4´,5,5´-hexachlorobiphenyl (PCB-153); 2,3,3´,4,4´,5-hexachlorobiphenyl (PCB-156); 3,3´,4,4´,5,5´-hexachlorobiphenyl (PCB-169); and 2,2´,3,4,4´,5,5´ heptachlorobiphenyl (PCB-180). The ratio of its components was designed to mimic

those found in biologically and environmentally relevant sources such as fish and human breast milk. The PHAH mixture contained 6001µg total PHAH/ml (see Table 1a for concentrations of individual components). This mixture was diluted with corn oil (Sigma Aldrich) to attain a concentration of 601 ug/ml (Stock solution A). Stock solution A was used to make eight serial dilutions (actual dose of total PHAHs for each dilution shown in parenthesis): 66% (396 ug/kg), 50% (300 ug/kg), 33% (198 ug/kg), 10% (60.1 ug/kg), 6.6% (39.7 ug/kg), 5.0% (30.0 ug/kg), 3.3% (19.8 ug/kg), and 1.0% (6.01 ug/kg).

Stock suspension B was prepared by combining 15.0 mg/ml thiram, 10.0 mg/ml pronamide, and 10.0 mg/ml mancozeb with corn oil (Table 1b). Stock B was diluted to make eight serial dilutions and the suspension was dosed at 5ml/kg, which resulted in dilutions and doses of 66% (115 mg/kg), 50% 87.5 mg/kg, 33% (57.7 mg/kg), 10% (17.5 mg/kg), 6.6% (11.5 mg/kg) , 5.0% (8.75 mg/kg), 3.3% (5.78 mg/kg), and 1.0% (1.75 mg/kg). All pesticide suspensions were stirred continuously during dosing to ensure homogeneity.

Animals: Female Long-Evans rats, 23 days old, were purchased from Charles River Laboratories (Raleigh, NC). Animals were received at the US EPA animal facility and allowed to acclimate for four days prior to dosing. Animal facilities were approved by the Association for Accreditation of Laboratory Animal Care and all procedures were approved in advance by the US EPA Institutional Animal Care and Use Committee. Animals were initially weighed two days prior to dosing and semi-randomly assigned to treatment groups such that animals in each dosing group had a similar mean weight. Two animals were housed per cage (45cm x 24cm x 20cm) with sterilized pine shaving

bedding (Beta Chips, Northeastern Products, Inc., Warrensburg, NY). Feed (Purina Rodent Chow 5001, Barnes Supply Co., Durham, NC) and tap water (Durham, NC) were provided *ad libitum*. Tap water was filtered through sand, activated charcoal and subsequently rechlorinated to 4-5 ppm Cl^- before use in the animal facility. A 12:12 light:dark photoperiod (light 0600hr-1800hr) was maintained and temperature and humidity was controlled at 22.0 ± 2.0^oC, and 50 ± 10%, respectively.

Animal Dosing

Individual Pesticide Dosing: Three separate groups of animals (n=8-16/group) were used to generate dose response curves for thiram, mancozeb, and pronamide. Dilutions of individual pesticides were administered to animals via oral gavage for four days. Animals in the control groups were dosed with corn oil. Doses of chemicals were adjusted daily based on animal weight. Tissue collection began 24 hours after the last dose, and the serum concentration of T_4 was evaluated by RIA.

Pesticide Mixture Dosing: Three groups of animals (n=8 per treatment) were exposed to a mixture of the three pesticides at dilutions of 50% or 5% of Stock B (87.5 and 8.75 mg/kg/day, respectively) or a corn oil control so dose additivity among the three pesticides could be evaluated. Animals were dosed via oral gavage for four days and serum was collected 24 hours after the last treatment. Dose additivity of PHAHs was confirmed using previous single chemical data (Crofton et al., 2005).

PHAH and Pesticide Mixture Dosing: Animals (n=8-16/group) were dosed with 66, 50, 33, 10, 6.6, 5.0, 3.3, and 1.0% dilutions of the PHAH and pesticide mixture or corn oil (control). Rats were dosed daily for four consecutive days via oral gavage. The

PHAH mixture was administered at 0900 hr at 1ml/kg/day and the pesticide mixture was dosed at 1100 hr at 5ml/kg/day. Each animal was weighed daily and doses of each mixture were adjusted according to body weight. In order to permit sufficient time for animal dosing and collecting tissue, five separate blocks of animals (16-26 animals/block; one block per week for five weeks) were used for exposures. This ensured that serum was collected at the same time of day as to minimize the influence of natural variation in hormone levels that occur throughout the day. Treatment groups were spread among blocks but not all blocks contained all treatments; each block had its own control group to allow for quantification of baseline T_4 and inter-block comparison. Fresh pesticide solutions were prepared each week. All solutions were stored in the dark at room temperature.

Hepatic enzyme assays: Microsomal liver fractions were prepared as previously described (DeVito et al., 1993). Hepatic EROD (a marker for CYP1A1 activity), PROD (a marker for CYP2B), and microsomal T_4-UDPGT activities were assayed for individual pesticides as described by Zhou et al, 2001.

Hormone Analysis: Trunk blood was collected from each animal following decapitation beginning 24 hours after the last day of dosing, and all samples were gathered between 0900hr and 1130hr. Blood was collected in serum separator tubes (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ) and allowed to clot on ice for a minimum of 30 minutes. Serum was separated by centrifugation at 4 ^{o}C, 3000 rpms for 25 minutes and stored at -80^{o}C until analysis.

Serum T_4 levels were measured by radioimmunoassay (Coat-A-Count Total T4, Diagnostic Products Corporation, Los Angeles, CA). Known calibration standards (0, 10, 40, 100, 160, and 240 ng/L of T_4), plasma samples, and quality controls (low, med, and high) were processed in provided antibody coated tubes. One additional standard of 5 ng/ml was included by making a 50:50 (by volume) mixture of 0 and 10 ng/l standards. Calibration standards were run in triplicate; serum samples and quality control were run in duplicate according to kit instructions. Variability between replicates was ≤ 5%. Serum T_4 concentrations were expressed relative to the amount of T_4 in control animals (% of control).

Evaluation of toxicity of mixture components: Dose-response data describing the impact of single chemicals on T_4 depletion was generated for the three pesticides and previously for all 18 PHAHs (Crofton et al, 2005). All group mean statistics are available as supplemental material. Individual dose response curves for each chemical (18 PHAHs and 3 pesticides) were fit with a sigmoidal function using Equation 1, and carried out by Origin software (OriginPro 7.5, Microcal Software Inc, Northampton, MA).

$$R = \frac{1}{1 + \left(\frac{ED50}{D} \right)^{\rho}}$$

Equation 1

In Equation 1, R represents the response to the chemical (% decrease of T_4), ED_{50} is the dose which elicits a 50% decrease in T_4, D is the dose of the chemical, and ρ is the slope

factor. The ED_{50} and ρ values calculated by Origin software were subsequently used in the mixture modeling.

Predictive modeling was calculated based on exposure to the mixture of three pesticides, mixture of 17 PHAHs (OCDF was excluded in all calculations as it did not display dose-responsive behavior at tested concentrations), and total mixture containing both the PHAHs and pesticide mixtures. In order to compare responses predicted by the models, both dose addition and effect addition were calculated for each mixture. Additionally integrated addition was calculated for the total mixture.

Dose Addition: Dose addition is often used to predict the response to mixtures of chemicals that act through the same mechanism of action. All chemicals predicted to be acting by dose addition are placed in one group, or "cassette." Predictions of dose addition are based on the ED_{5o} and dose for each chemical and the average ρ value for all chemicals in the same cassette. The dose addition equation used was

$$R = \frac{1}{1 + \frac{1}{\left(\sum_{i=1}^{n} \frac{D_i}{ED50_i} \right)^{\rho'}}}$$

Equation 2

where R is the response to the mixture, D_i is the dose of chemical, $ED50_i$ is the chemical dose that results in a 50% decrease in T_4 and ρ' is the average slope factor of all chemicals grouped with the same mechanism of action.

Effect Addition: Effect addition was used to calculate the response to mixtures containing chemicals acting through different mechanisms of action but with a common response—decreased circulating T_4 concentrations. Effect addition places each component of the mixture in a separate cassette, and prediction of effect is based on the $ED_{50,}$ dose, and ρ for each individual chemical. The equation used to calculate effect addition was

$$R = 1 - \prod_{i=1}^{n} (1 - R_i)$$

Equation 3

where R is the response to the mixture, and R_i represents the response to the chemical in cassette *i* (which is calculated for each chemical individually using Equation 1).

Integrated Addition: Integrated addition can be used to predict the response to a mixture when chemical modes of action are well understood, and have similar and dissimilar modes that affect a common endpoint. To calculate integrated addition, chemicals were grouped based on mode of action. The response to the PHAHs was calculated in one cassette, based on a similar mode of action: upregulation of hepatic clearance of T_4 (Shelby and Klaassen, 2006). The response to the pesticides was calculated as a second cassette based on a shared mode: decreased hormone synthesis (Doerge and Takazawa, 1990). Conceptually, dose addition is calculated within cassettes and effect addition is calculated between cassettes (Figure 1). The integrated addition equation described by Olmstead and LeBlanc, 2005 that combines the concepts of dose and effect addition and was used for this prediction is

$$R = 1 - \prod_{I=1}^{N} \left\{ 1 - \frac{1}{1 + \frac{1}{\left(\sum_{i=1}^{n} \frac{C_i}{ED50_i} \right)^{\rho_{avg,I}}}} \right\}$$

Equation 4

Model Analysis: The model used in this study was not designed to provide terms to determine statistical error of the predicted responses. Therefore, deviations between predictions and empirical data were determined by plotting model predictions against empirical data and comparing predictions to a line depicting a 1:1 correlation between the data and model predictions. Data points falling to the right of the 1:1 correlated line signify an overestimation of the effects of exposure (ie, empirical data > model prediction). Those points falling to the left of the 1:1 correlation show predictions that underestimate the effects exposure (ie, empirical data < model prediction). Empirical data was judged to significantly deviate from model predictions when the standard deviation associated with the data did not overlap with the line depicting a 1:1 correlation between the empirical data and modeled response. The results of all three models predicting the response to the PHAH and pesticide mixture were compared to determine which most closely predicted the mean values of the empirical data. The magnitude and direction of deviation and mean absolute value of deviation from the 1:1 correlation were determined.

Results

Evaluation of individual chemical components and PHAH or pesticide mixture: Exposure to the individual pesticides produced a dose-dependent decrease in the circulating levels of T_4 (Figure 2). No induction of EROD, PROD or T_4-UGT was observed for thiram and mancozeb (data not shown). Small increases (1-fold) in T_4-UGT were observed for pronamide at dose levels 2-4 times higher than those that resulted in decreases in T_3 and T_4 (data not shown). The ED_{50} and slope parameters for use in the model were determined by fitting single pesticide data with the previously described sigmoidal function (Table 2). The empirical T_4 data did not significantly deviate from predictions of dose addition and effect addition at 50% and 5.0% (Figure 3). Both models well predicted the effects of the pesticide mixture.

Single chemical data describing T_4 response was previously generated for each component of the PHAH mixture (Crofton et al, 2005). The ED_{50} and slope parameters for each chemical were determined (Table 2). Both the dose addition model and effect addition model predicted the response to the PHAH mixture between doses of 0 ug/kg and 200 ug/kg (Figure 4). Thyroxine (T_4) concentrations at the three highest doses (667, 1335, and 2003 ug/kg) were not well predicted by either model, and both underestimated the effects of these high exposures. This agrees with the predictions of a flexible single-chemical-required method applicable to chemicals with differing dose thresholds and maximum-effect asymptotes previously reported for the same mixture of PHAHs (Crofton et al., 2005). The total doses of PHAHs used in the present study were 397 ug/kg or less, and these doses are not likely to deviate from dose additivity. Dose additivity between chemicals is assumed when grouping similar chemicals for the

purpose of generating an integrated addition prediction, as was performed for the total mixture of PHAHs and pesticides.

Total Mixture with PHAHs and Pesticides: No mortality was observed during exposure to the mixture of PHAHs and pesticides and the only visible sign of toxicity occurred in the highest dose group where animals gained an average of 35% (approximately 10g) less weight than controls (data not shown). Model predictions based on dose addition, effect addition, and integrated addition were compared to the empirical data (Figure 5a). Significant deviation from predictions (standard deviations that did not over lap the line depicting a 1:1 correlation) were observed in the following models and doses: effect addition, 3.3 and 50%; dose addition, 66%; integrated addition, 3.3% (Figure 5 b,c,d). The results of all three models were compared to determine which model most closely predicted the mean value of the empirical data for each dose group. The magnitude and direction of deviation and absolute value of deviation from the 1:1 correlation was determined. In general, the integrated addition model predictions most closely predicted the observed response to the chemical mixture (Figure 6). However, both the predictions of the integrated addition and dose addition models deviated less from the mean of the empirical data than the effect addition model in all but the 66% dose group. The effect addition model showed a trend of overestimation of the effects of exposure (empirical data > model prediction) to the mixture by 6-21 points. The mean absolute deviation from the empirical data was 10.8 points for effect addition, 7.3 for dose addition, and 6.5 for integrated addition.

Discussion

This research shows that the effects of a chemical mixture that disrupts thyroid hormone concentrations through two distinct mechanisms can be best represented by dose addition and integrated addition models. While the effect addition model predicted well for most points, the empirical data tended to be greater than the model predictions. Thus, the effect addition modeled predictions tended to overestimate the effects of exposure to the mixture.

The overestimation of effects produced by the effect addition model is likely due poor sigmoidal fits of several individual chemicals. A few chemicals (most notably, pronamide and PCDD) resulted in low ρ values and did not well fit all points in the dose response curve. This could cause an overestimation of effects when calculating the response to individual chemicals to be used in effect addition. Chemicals with the same mechanism of action are assumed to have similar ρ values. Averaging ρ values, as is done in dose addition and within cassettes of integrated addition lessens the potential overestimations of these chemicals with low ρ values. Effect addition bases the predicted responses of individual chemicals on their ρ values, thus leading to response overestimations of some compounds. The integrated addition model and dose addition model both closely predicted the same trends. This is likely due to similar ρ values in the pesticides and PHAHs.

Animals exposed to the highest dose of the PHAH and pesticide mixture (66%) gained significantly less weight than control animals. This is likely due to the thiram component; it has been shown to reduce weight gain in other exposed animals (Hasegawa et al, 1988). Additionally, the present evaluation of the individual pesticides, thiram

dosed at 50 mg/kg/day showed a similar decrease in weight gain, which is approximately the highest dose used in the mixture studies. The concentrations of T_4 at this dose were still consistent with predictions of integrated addition so it is not likely that reduced weight gain caused a significant effect on T_4. However, dose addition significantly overestimated the response to the mixture at exposure to 66% of the stock solution. These changes in body weight gain are early indicators of systemic toxicity, which may be impact the ability of the models to accurately predict hormone changes.

The mechanism of action of thiram and pronamide are not completely understood but are likely similar to other TPO inhibitors. Chinese hamster ovary cells transfected with the human TPO gene show no effect of thiram on TPO inhibition when using iodination of a Glu-Tyr-Glu peptide and oxidation of guiacol as endpoints (Marinovich et al, 1997). However, TPO is complex enzyme that functions beyond iodination, such as the coupling of monoiodinated tyrosine (MIT)/ diiodinated tyrosine (DIT) to make T_3 and T_4 (Taurog et al., 1996). Thus while thiram may not inhibit iodination or oxidation of guiacol *in vitro*, it may inhibit those processes *in vivo* or inhibit other functions of this enzyme that are involved in TH synthesis. Other data indicate more similarities between thiram and pronamide and other TPO inhibitors than thyroid hormone disruptors that induce UGTs or inhibit deiodination. Exposure to thiram and pronamide results in significant decreases in T_4 in rats in a pattern similar to other TPO inhibitors such as mancozeb (Hurley et al., 1998; Hedge et al., 2002). In studies of thiram, there was no induction of T_4-glucuronidiation or enzymatic markers of CYP induction (EROD and PROD). Only small increases (1-fold) in UGTs were observed for pronamide at doses 2-4 times higher than those that resulted in hormone decreases. Thus thiram and pronamide

are not likely to decrease THs through hepatic effects at the current tested doses. Additionally, they are not likely to be specific inhibitors of deiodinases because exposure does not cause a significant increase in circulating T_4, a hallmark of FD&C Red No.3, a specific deiodinase inhibitor (Capen 1997). Therefore, for the purpose of this study, thiram and pronamide were placed into the TH synthesis inhibitor group.

Research is beginning to focus on the effect of complex mixtures of chemicals that target the same physiological endpoint but do so through various mechanisms of action. Altenburger et al (2004) used a model that calculates the combined effect of concentration addition and effect addition to describe the response of organisms to a 10-compound mixture of toxicants found in sediment. Rider and LeBlanc (2005) incorporated terms for toxicokinetic interactions into the integrated dose and effect addition model to develop an integrated addition and interaction model (IAI). This approach adds coefficient of interaction terms to describe certain components of the mixture that affect the potency of others. Predictions produced by this method were far superior for their mixture of organophosphates and a P450 inhibitor to those that only used dose addition, effect addition, or an integrated addition. Models show promise for accurately predicting response to chemical mixtures especially when modes of action for individual chemicals are understood. In this study, calculating the contribution of TH synthesis inhibitors and TH clearance enhancers by integrated addition resulted in predictions that were generally closer to the empirical data than dose addition or effect addition alone.

Humans are exposed to mixtures of thyroid hormone disrupting chemicals through foods, drinking water, and the environment so it is important to understand the

possible implications of exposure. Receptors for thyroid hormone are present in most tissues including the heart, bone, fat, liver, pituitary, liver, and brain where THs play an important role in metabolism, growth, and development (Yen, 2001). Hypothyroidism in developed animals is characterized by low THs, elevated TSH, decreased metabolism and an enlarged thyroid gland. High levels of TSH stimulated by low circulating THs have been linked with thyroid tumors in rodents (Hard, 1998). Additionally, low circulating concentrations of THs in developing animals are correlated with neurological problems and structural brain defects (Koibuchi and Chin, 2000). Howdeshell (2002) reviewed the importance of thyroid hormone in brain development and found it to be vital beginning from the time of oocyte maturation and throughout gestation. It is clear that environmental chemicals that affect TH levels in the mother or developing fetus may result in permanent alterations. Human *in utero* exposure to thyroid disrupting chemicals might be linked with neurodevelopmental and behavioral problems including attention deficit hyperactivity disorder and autism (Colborn, 2004). Many studies clearly define the effects of single chemicals on thyroid hormone disruption; however, because chemicals can act additively, focusing on only a single environmental chemical may underestimate the true risk of hormone disruption. Combinations of common environmental contaminants should be tested to fully understand their toxicity to the thyroid hormone system.

A variety of environmental xenobiotics individually disrupt thyroid hormone concentrations and mixtures of chemicals with the same mode of action can act additively to decrease T_4. This research helps to present a clearer understanding of how T_4 levels are affected when TH synthesis is inhibited and hepatic microsomal enzymes are induced

by chemical mixtures. Understanding the biological effects of mixtures creates a better ability to predict potential issues in human and environmental health.

Table 1. **Mixture components and dose of individual PHAHs and pesticides administered at the highest doses.** a) Components and amounts of PHAHs in the mixture. Solution was dosed at 1ml/kg/day. The highest dose used in this study contained 66% of the stock solution. b) Components and amounts of pesticides in the mixture. Solution was dosed at 5ml/kg/day. The highest dose used in this study contained 66% of the stock solution.

1a)

Doses and Components of PHAH Mixture

Chemical	Stock Solution A (ug/kg)	66% (ug/kg)
TCDD	0.00390	0.00257
PCDD	0.00390	0.00257
TCDF	0.00570	0.00376
1-PCDF	0.00180	0.00119
4-PCDF	0.00780	0.00515
OCDF	0.0195	0.0129
PCB-28	23.6	15.6
PCB-52	46.6	30.7
PCB-77	0.600	0.396
PCB-101	46.1	30.4
PCB-105	23.0	15.2
PCB-118	114	75.5
PCB-126	0.183	0.121
PCB-138	114	75.4
PCB-153	115	75.7
PCB-156	3.93	2.59
PCB-169	0.120	0.0792
PCB-180	113	74.8
Total	601	397

Table 1 (Continued)

1b)

Doses and Components of Pesticide Mixture

Chemical	Stock Solution B (mg/kg)	66% (mg/kg)
Pronamide	50.0	33.0
Mancozeb	50.0	33.0
Thiram	75.0	49.5
Total	175.0	115.5

Table 2. ED_{50} and ρ values for individual components of the mixture.

ED_{50} and ρ values for Mixture Components

Chemical	ED_{50}	ρ
Thiram	250	1.31
Mancozeb	259	1.19
Pronamide	116	0.50
TCDD	1.00	1.44
PCDD	4.37	0.46
TCDF	24.4	0.79
1-PCDF	100	1.49
4-PCDF	90.0	1.16
OCDF	N/A*	N/A*
PCB-28	90000	1.59
PCB-52	90000	0.74
PCB-77	2025	0.95
PCB-101	11900	0.91
PCB-105	1580	2.21
PCB-118	1980	2.32
PCB-126	8.23	1.24
PCB-138	14990	0.87
PCB-153	24500	1.06
PCB-156	1330	0.78
PCB-169	506	1.23
PCB-180	54900	2.45

* OCDF was not dose responsive at tested concentrations

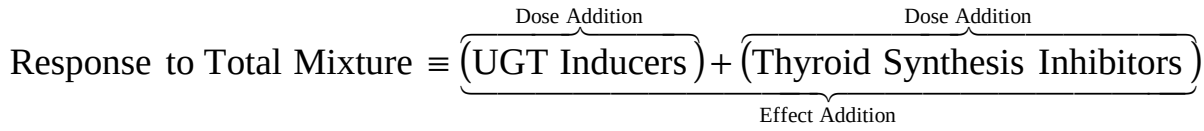


Figure 1. **Schematic depiction of integrated addition model.** The contributions of UGT inducing chemicals (PHAHs) and thyroid synthesis inhibitors (pesticides) were calculated by dose addition. The total response to the mixture with PHAHs and pesticides was calculated using the effect addition model.

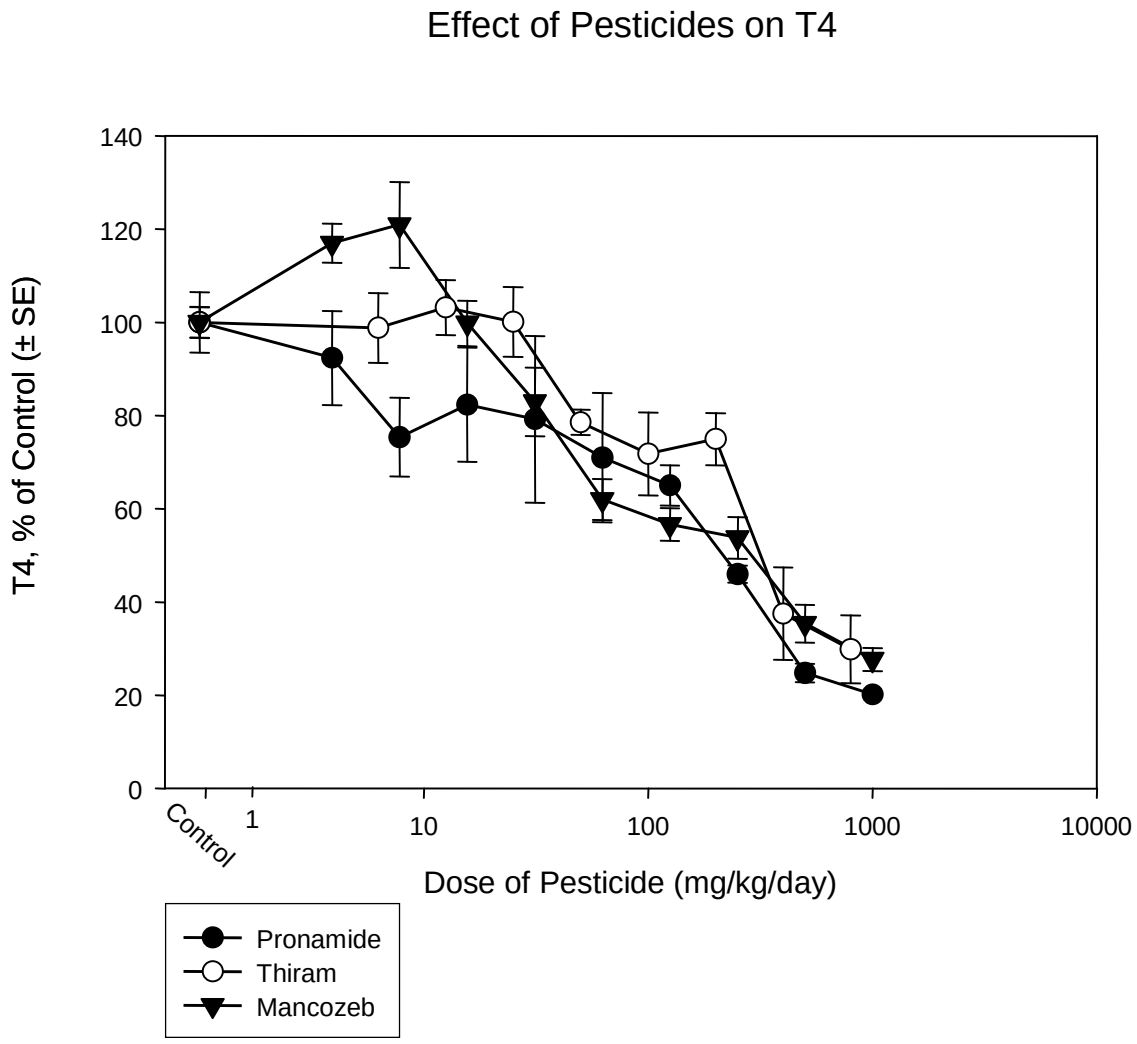
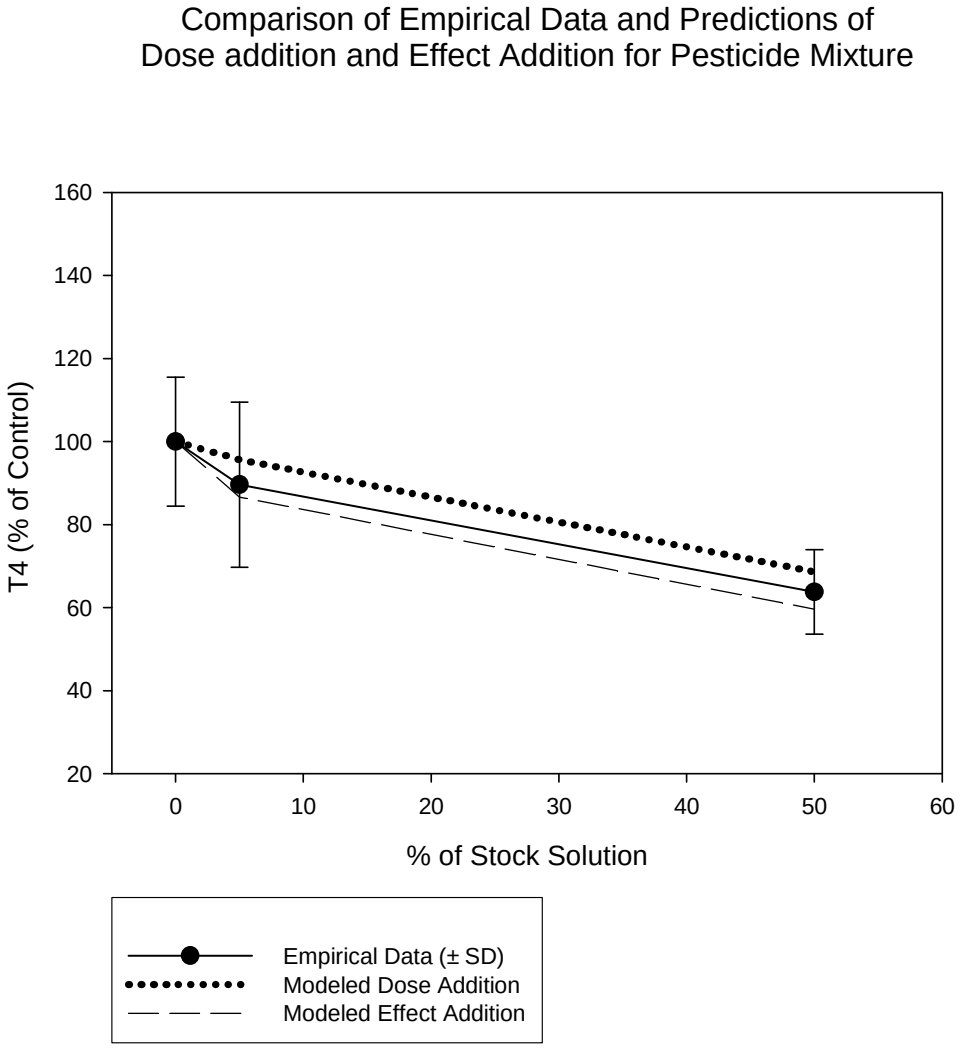


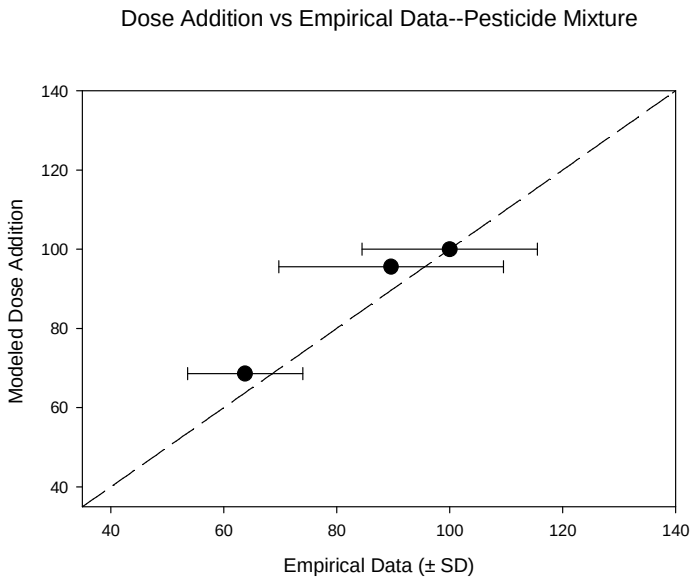
Figure 2. Effect of individual pesticides on serum T_4 following a four-day exposure. Data are presented as percent of controls (±SE). Control values were considered to have 100% of circulating T_4

Figure 3. Predictive modeling of pesticide mixture. a) Comparison of dose addition and effect addition models to empirical data of pesticide mixture. **b)** Deviation between empirical data and effect addition model. **c)** Deviation between empirical data and dose addition model. The dashed line represents 1:1 congruence between model predictions and empirical data.

3a)


Comparison of Empirical Data and Predictions of
Dose addition and Effect Addition for Pesticide Mixture
T4 (% of Control)
160
140
120
100
80
60
40
20
0
10
20
30
40
50
60
% of Stock Solution
Empirical Data (± SD)
Modeled Dose Addition
Modeled Effect Addition

3b)


Dose Addition vs Empirical Data--Pesticide Mixture
Modeled Dose Addition
Empirical Data (± SD)
140
120
100
80
60
40
40
60
80
100
120
140

3c)

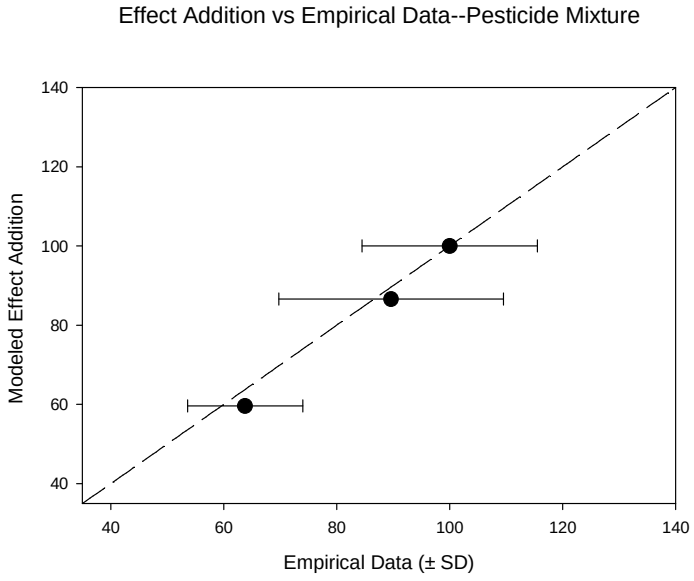
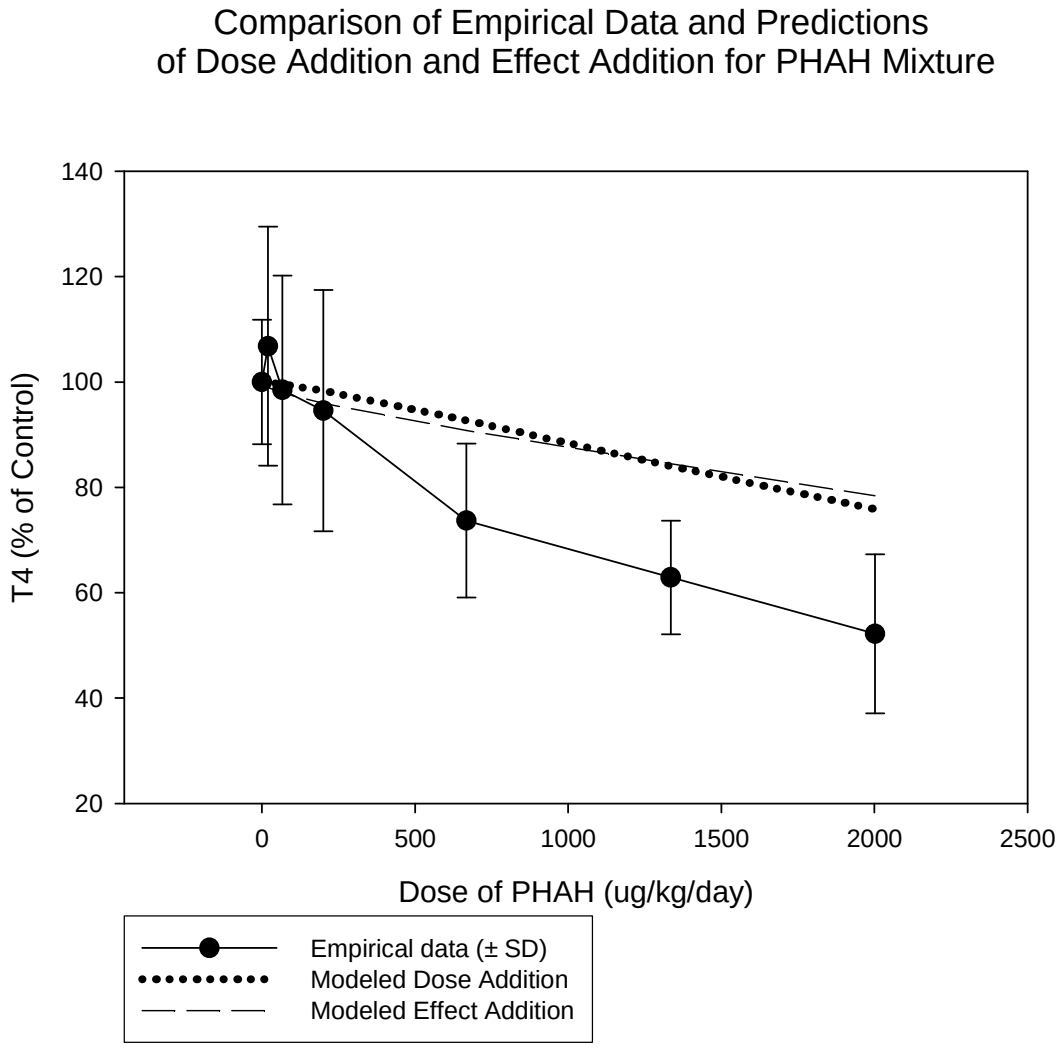
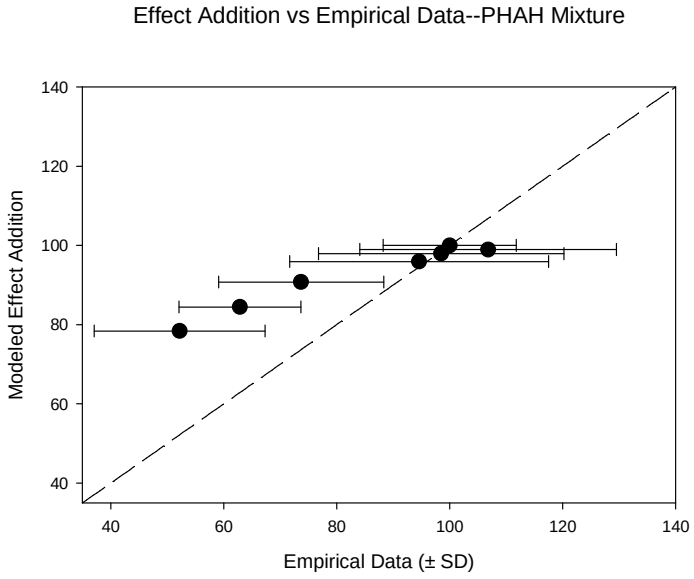

Effect Addition vs Empirical Data--Pesticide Mixture
Modeled Effect Addition
Empirical Data (± SD)
140
120
100
80
60
40
40
60
80
100
120
140

Figure 4. Predictive modeling of PHAH mixture. a) Comparison of dose addition and effect addition models to empirical data of PHAH mixture from Crofton et al, 2005. **b)** Deviation between empirical data and effect addition model. **c)** Deviation between empirical data and dose addition model. The dashed line represents 1:1 congruence between model predictions and empirical data.

4a)


Comparison of Empirical Data and Predictions
of Dose Addition and Effect Addition for PHAH Mixture
T4 (% of Control)
140
120
100
80
60
40
20
0
500
1000
1500
2000
2500
Dose of PHAH (ug/kg/day)
Empirical data (± SD)
Modeled Dose Addition
Modeled Effect Addition

4b)


Effect Addition vs Empirical Data--PHAH Mixture
Modeled Effect Addition
Empirical Data (± SD)
140
120
100
80
60
40
40
60
80
100
120
140

4c)

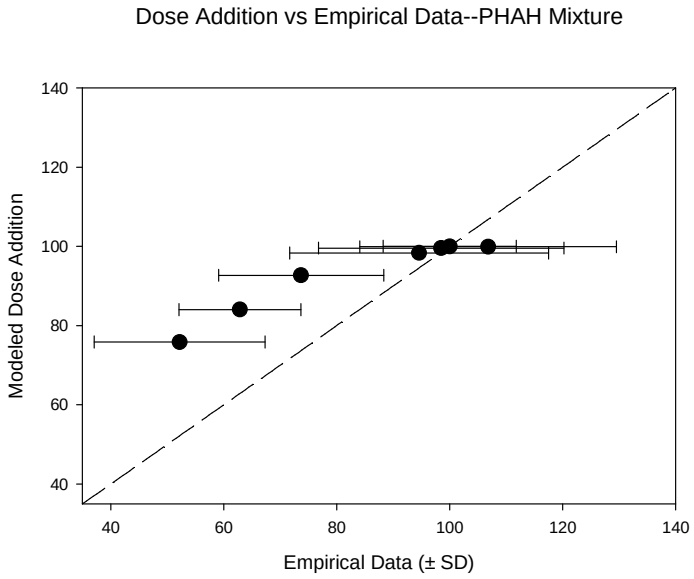
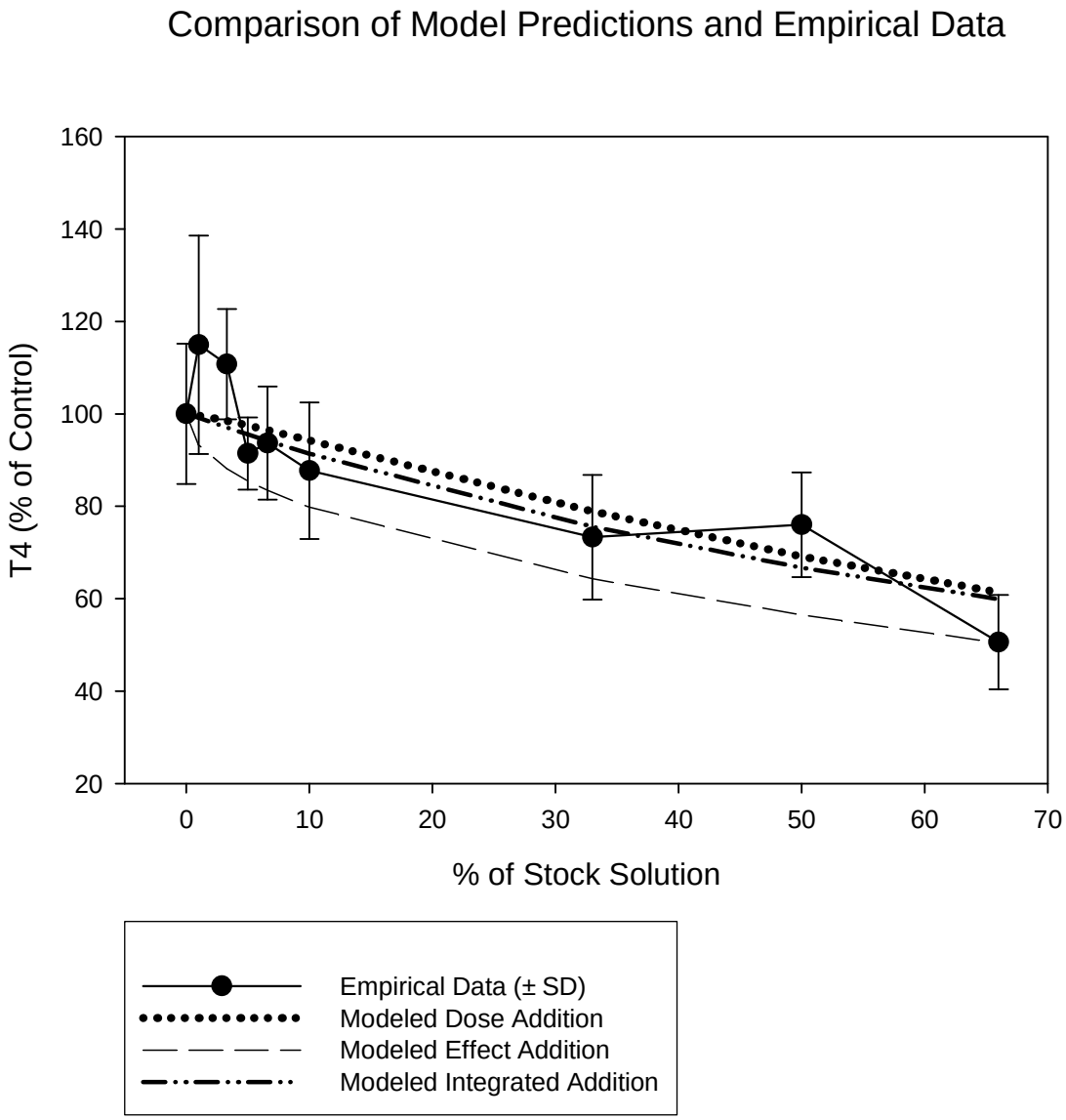
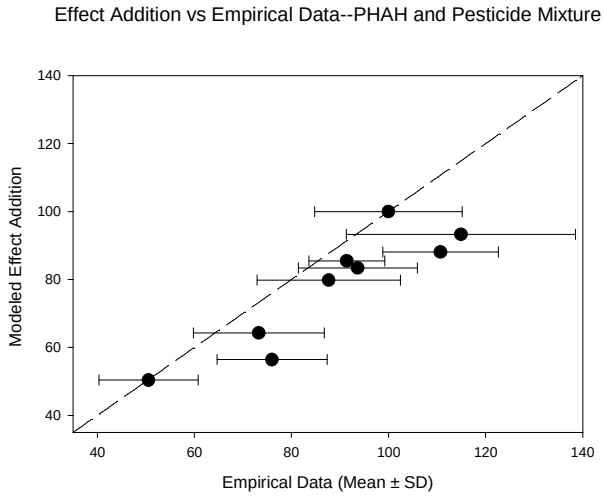

Dose Addition vs Empirical Data--PHAH Mixture
Modeled Dose Addition
Empirical Data (± SD)
140
120
100
80
60
40
40
60
80
100
120
140

Figure 5. Predictive modeling of PHAH and pesticide mixture. a) Comparison of dose addition, effect addition, and integrated addition models to empirical data of PHAH and pesticide mixture . **b)** Deviation between empirical data and effect addition model. **c)** Deviation between empirical data and dose addition model. The dashed line represents 1:1 congruence between model predictions and empirical data.

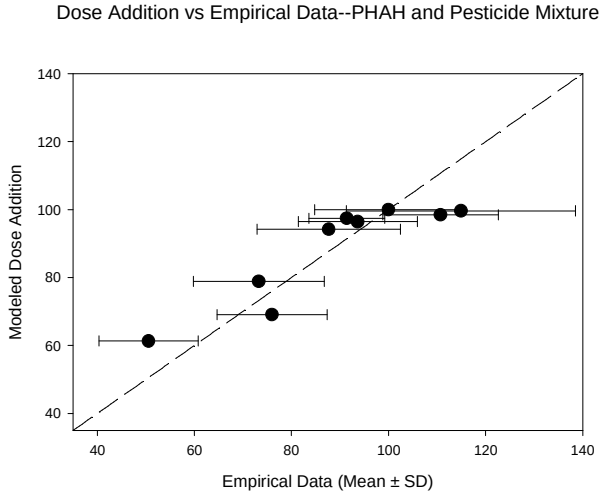
5a)


Comparison of Model Predictions and Empirical Data
T4 (% of Control)
160
140
120
100
80
60
40
20
0
10
20
30
40
50
60
70
% of Stock Solution
Empirical Data (± SD)
Modeled Dose Addition
Modeled Effect Addition
Modeled Integrated Addition

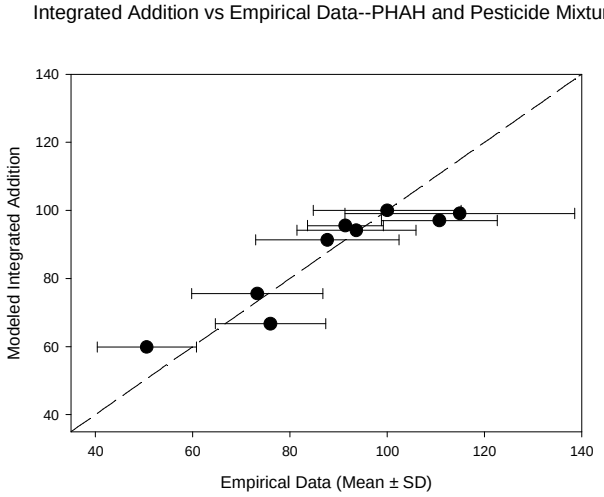
5b)


Effect Addition vs Empirical Data--PHAH and Pesticide Mixture
Modeled Effect Addition
Empirical Data (Mean ± SD)
140
120
100
80
60
40
40
60
80
100
120
140

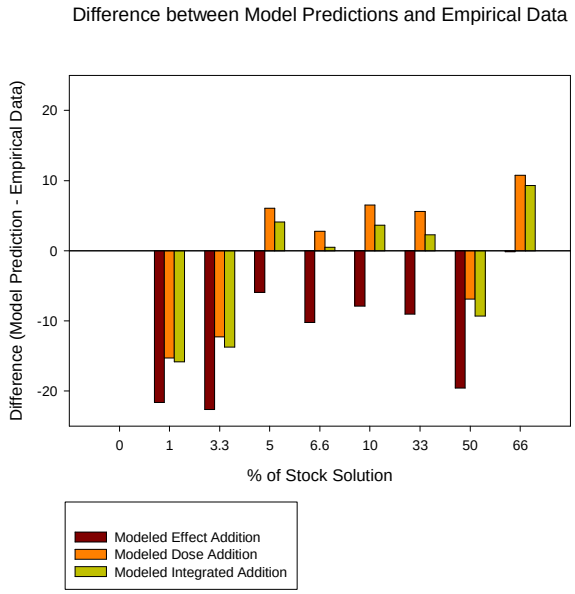
5c)


Dose Addition vs Empirical Data--PHAH and Pesticide Mixture
Modeled Dose Addition
Empirical Data (Mean ± SD)
140
120
100
80
60
40
40
60
80
100
120
140

5d)


Integrated Addition vs Empirical Data--PHAH and Pesticide Mixture
Modeled Integrated Addition
Empirical Data (Mean ± SD)
140
120
100
80
60
40
40
60
80
100
120
140

6a)



6b)

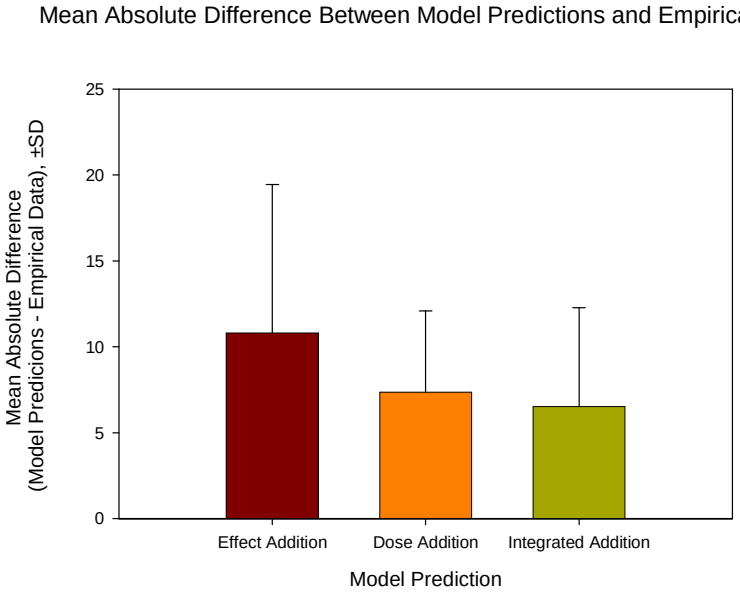


Figure 6. Deviation between model predictions and empirical data. a) Magnitude and direction of deviation between model predictions and empirical data. **b)** Mean absolute deviation between model predictions and empirical data.

CHAPTER III

The mRNA Levels of Certain Thyroidal Genes May Not Be a Useful Biomarker of Exposure to Thyroid Hormone Synthesis Inhibiting Pesticides

Abstract

Various xenobiotics interfere with the synthesis, transport, and clearance of thyroid hormones. Specifically, two fungicides, thiram and mancozeb and the herbicide pronamide inhibit hormone production in the thyroid gland. Exposure to these chemicals produces a decrease in circulating T_4 concentrations. These decreases may be associated with altered expression of genes in the thyroid gland (PAX-8, Hex, Tg, NIS, or TPO). It was hypothesized that expression of these genes would increase following exposure to the pesticides. Female Long-Evans rats were exposed for four days to two dilutions of each of the following mixtures: three pesticides (thiram, mancozeb, and pronamide); 18 PHAHs (dioxins, furans, and PCBs); a combination of the three pesticides and 18 PHAHs. Thyroids were collected and rtPCR was used determine if alteration of key thyroid genes occurred as a result of TH synthesis inhibition. Thyroidal expression of PAX-8, Hex, Tg, NIS, and TPO mRNA was not significantly different than corn oil treated control animals. The expression patterns of the genes tested in this study do not provide a useful biomarker of short term exposure to the tested doses of TH synthesis inhibiting pesticides.

Introduction

A variety of environmental chemicals interfere with the synthesis, transport, and clearance of thyroid hormones (THs) (Brucker-Davis, 1998; Boas et al, 2006). Altered production or clearance leads to a similar endpoint-- decreased serum thyroxine (T_4) concentrations. This general endpoint is important because low levels of T_4 have been associated with neurological damage and structural brain defects (Koibuchi and Chin, 2000), developmental impairment (Howdeshell, 2002), and behavioral problems (Colborn, 2004).

Clearance of thyroid hormones in the rat is mediated by specific UDP-glucuronosyl transferase (UGT) enzymes in the liver and they can be upregulated by PCBs, dioxins, and furans (Shelby and Klaassen, 2006; Schuur et al, 1997). These UGT enzymes facilitate the catabolism and excretion of many xenobiotics and endogenous hormones (Kretschmer and Baldwin, 2005). The UGT 1A1 and 1A6 isoforms are responsible for metabolizing T_4 and these mRNA levels increase when exposed to various chemicals, and the gene profiles can be used as biomarkers of exposure.

Synthesis of T_4 occurs within the thyroid gland by coupling tyrosine residues with iodine, and this process is catalyzed by the thyroid peroxidase (TPO) enzyme (Larsen et al, 2003; Zoeller et al, 2007). The fungicides thiram and mancozeb and the pesticide pronamide are thought to inhibit the TPO enzyme and thereby reduce levels of serum T_4.

A variety of genes are involved in the regulation and function of the thyroid, but the most well studied are involved in thyroid hormone production. These include haematopoietically expressed homeobox (HEX), paired box gene 8 (PAX-8), thyroid transcription factor 1 and 2 (TTF-1, TTF-2), thyroid hormone receptor (TSHr), sodium

iodine symporter (NIS), thyroglobulin (Tg), thyroid peroxidase (TPO) and dual oxidase 1 and 2 (DUOX 1, DUOX 2) (Lacroix et al, 2006).

The relationship between thyroidal genes is complex and not yet completely understood, but their expression seems to be partially dependent on one another. Following direct interaction of PAX-8 and TTF-1 the complex binds to the promoter regions of TPO, Tg, and TSHr genes to result in increased transcription (Di Palma et al, 2003; Lacroix et al, 2006). Thyroglobulin causes negative feedback on TTR-1 and PAX-8 and also suppresses the mRNA levels of NIS, TPO, Tg, and the TSH receptor (Suzuki et al, 1998; Suzuki et al, 1999). Hex has been reported to be a repressor of the Tg promoter and thus eliminates the activating effects of TTF-1 and PAX-8 to stimulate transcription (Martinez et al, 2000; Pellizzari et al, 2000).

Evidence suggests that chemicals which directly inhibit TPO lead to an increase in certain mRNA. Thyroid cells exposed to MMI and PTU, antithyroid drugs that inhibit TPO, resulted in TSH-independent elevated levels of mRNA coding for TPO (Sugawara et al., 1999; Isozaki et al., 1991) and Tg (Leer et al., 1991). Thus, in addition to reducing T_4 production, binding to the TPO enzyme may cause a TSH independent gene transcription event. Gene expression patterns in the thyroid gland may serve as useful biomarkers to determine endocrine disruption and pathological endpoints (Glatt et al., 2005). The purpose of this study is to determine if thyroidal mRNA levels of PAX-8, Hex, Tg, NIS, and/or TPO can be utilized as biomarkers to indicate animal exposure to chemical inhibitors the TPO enzyme. The development of such biomarkers could be a useful tool in screening chemicals for thyroid hormone disrupting activity.

Currently, rats were exposed to a mixture of TH synthesis inhibiting chemicals (pesticides), UGT inducing chemicals (PHAHs), and a mixture of both pesticides and PHAHs. It was hypothesized that exposure to TH synthesis inhibitors would result in an increase in expression of the tested thyroid genes. Thyroids were collected and rtPCR was used to measure mRNA of the target genes to detect alterations in gene expression.

Materials and Methods

Chemicals: A mixture of 18 polyhalogenated aromatic hydrocarbons (PHAHs) consisting of dioxins, furans, and PCBs was previously synthesized for Crofton et al (2005). The PHAH mixture was originally obtained from Cambridge Isotope Laboratories and contained 6001μg/ml of PHAHs. This mixture was diluted with corn oil (Sigma Aldrich) to produce a concentration of 601 ug/ml (Stock solution A). Stock solution A was diluted with corn oil (Sigma Aldrich) to two solutions: 5.0% (30.0 ug/kg) and 50% (300 ug/kg) of stock solution A (Table 1a). PHAHs decrease serum levels of T_4 but do so by increasing metabolism and clearance of the hormones (Shelby and Klaassen, 2006); this mixture was used to differentiate any effects due to general T_4 decrease rather than that primarily due to TH synthesis inhibition.

Stock suspension B was prepared by combining 15.0 mg/ml thiram, 10.0 mg/ml pronamide, and 10.0 mg/ml mancozeb with corn oil (Table 1b). Stock B was diluted to make two serial dilutions and the suspension was dosed at 5ml/kg, which resulted in dilutions and doses of 5.0% (8.75 mg/kg) and 50% (87.5 mg/kg). All pesticide suspensions were stirred continuously during dosing to ensure homogeneity.

Animals and Dosing: Female Long-Evans rats, 23 days old, were purchased from Charles River Laboratories (Raleigh, NC). Animals were received at the US EPA animal facility and allowed to acclimate for four days prior to dosing. Animal facilities were approved by the Association for Accreditation of Laboratory Animal Care and all procedures were approved in advance by the US EPA Institutional Animal Care and Use Committee. Animals were initially weighed two days prior to dosing and semi-randomly assigned to treatment groups such that each dosing group had a similar mean weight. Two animals were housed per cage (45cm x 24cm x 20cm) with sterilized pine shaving bedding (Beta Chips, Northeastern Products, Inc., Warrensburg, NY). Feed (Purina Rodent Chow 5001, Barnes Supply Co., Durham, NC) and tap water (Durham, NC) were provided *ad libitum*. Tap water was filtered through sand, activated charcoal and subsequently rechlorinated to 4-5 ppm Cl^- before use in the animal facility. A 12:12 light:dark photoperiod (light 600hr-1800hr) was maintained and temperature and humidity was controlled at 22.0 ± 2.0^oC, and 50 ± 10%, respectively.

Seven groups of rats (n=8/group) were dosed for four days via oral gavage with the following solutions: the mixture of three pesticides at 5 and 50%; the mixture of 18 PHAHs at 5 and 50%; a combined mixture of the three pesticides and 18 PHAHs at 5 and 50%; and a corn oil control. All solutions were stored in the dark at room temperature between treatments. The PHAH mixture was administered at 0900hr at 1ml/kg/day, and the pesticide mixture was dosed at 1100hr at 5ml/kg/day. Because animals dosed with the total mixture (PHAHs and pesticides) were dosed twice, animals that were only PHAHs or pesticides were dosed with corn oil in place of the other mixture component.

Thyroids and trunk blood were collected from each animal following decapitation beginning 24 hours after the last day of dosing. Thyroids were placed in RNAse-free tubes, weighed, promptly frozen in liquid nitrogen, and stored at -80^{o}C until processing. All tissues were gathered between 0900hr and 1130hr.

RNA Extraction and Quantification: Total RNA was extracted from each thyroid according to instructions of an RNeasy Mini Kit (Qiagen, Valencia, CA). The concentration of RNA for each sample was quantified using 1ul of sample on a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE). Each sample was run in duplicate and the average concentration of RNA was calculated. Samples were then diluted to 100ng/ul for determination of RNA integrity. The analysis of integrity was determined using the Agilent RNA 6000 Nano Kit and Agilent 2100 Bioanalyzer (Agilent Technologies, Inc.) according to kit instructions. The total RNA assay detects ribosomal RNA (18s and 28s) and calculates a ratio of the components to determine if degradation of RNA has occurred. Each RNA sample with appropriate integrity was then diluted with RNAse free water to 25ng/ul for rtPCR analysis. Diluted samples were stored at -80^{o}C until further analysis.

Real Time PCR: Gene probes for five thyroid genes of interest and a control were purchased as Taqman Gene Expression Assays (Applied Biosystems, Foster City CA). The chosen genes were PAX-8 (Rn00579743_m1), Hex (Rn00577750_m1), TPO (Rn00571159_m1), Tg (Rn00578496_m1), NIS (Rn00583900_m1); β-actin (Rn00667869) was selected as a control. Gene probes were prepared for PCR use by

mixing with TaqMan One-Step RT-PCR Master Mix Reagents (Applied Biosystems) according to instructions. The completed mixture consisted of master mix, a multiscribe and RNase inhibitor, one gene probe, and RNAse free water.

Samples and gene probes were loaded onto a 384 well plate at 4^{o}C. Each reaction contained 5ul of RNA (125ng) and 15ul of master mix components/gene probe. Samples were randomized across four 384-well plates and the six gene probes were randomized across each plate. Real time PCR was carried out on a 7900HG Sequence Detection System (Applied Biosystems).

Data Analysis: Changes in target gene expression were normalized to β-actin and the relative change in expression was quantified using the $2^{-\Delta\Delta C}{}_{T}$ method (Livak and Schmittgen, 2001). Data was analyzed by one way analysis of variance and significance was determined as $p \leq 0.05$.

Results

No mortality or obvious signs of toxicity were observed during exposure to any of the treatments. Previous analysis of serum by RIA indicated that T_4 levels decreased as a result of exposure to the tested chemicals (Chapter II, Figures 4a, 5a, and 6a). Exposure to three pesticides (5% and 50%), 18 PHAHs (5% and 50%), and a mixture of the three pesticides and 18 PHAHs (5% and 50%) did not result in a significant change in the expression of PAX-8 ($F=1.41$, $p=0.23$), Tg ($F=0.691$, $p=0.66$), Hex ($F=0.46$, $p=0.84$), NIS ($F=$, $p=$)or TPO ($F=0.73$, $p=0.62$) (Figure 1).

Discussion

This study suggests that the mixture of TH synthesis inhibiting pesticides does not cause a change in the expression of the genes tested (PAX-8, HEX, Tg, NIS, or TPO). Further, a circulating decrease of T_4 by the PHAH mixture or complex mixture of both PHAHs and pesticides does not cause a significant change in gene expression for the thyroid genes over the course of a four day exposure. It does not appear that these genes can be used for biomarkers when exposed to the doses or specific chemicals used in this study.

Short term studies such as this do not result in a decrease in the level of TSH (Zhou et al, 2000) which could be the key reason that no change in mRNA was observed. Many current studies indicate that gene expression is TSH dependent, but some previous studies suggest otherwise. Thyroid cells in an *in vitro* system exposed to MMI or PTU for 24 hours showed increases in Tg mRNA levels independent of TSH (Isozaki et al, 1991; Leer et al, 1991). However, PTU and MMI are strong inhibitors of the TPO enzyme and the concentrations that produced these results were higher than those required for complete inhibition of the enzyme (Leer et al, 1991). In contrast, rats in the current study were exposed to relatively lower doses of TH synthesis inhibitors that produced only a 50% decrease in T_4. Thus it is likely that the previously observed mechanism of TSH-independent Tg production did not occur in this case. Animals exposed to the mixture of pesticides experienced a decrease in T_4, but not a cessation of production that would result from complete inhibition of TPO. Furthermore, no information could be found to indicate that these TSH independent increases in Tg occur in *in vivo* systems. Sugawara et al (1999) showed a TSH independent increase in TPO

mRNA and enzyme activity following *in vitro* treatment with therapeutic levels of PTU and MMI. The present study did not show evidence that the pesticides could produce a similar effect at the experimental doses tested.

Though it is possible to increase the levels of thyroid mRNA genes without increasing TSH, it did not occur in this instance. Previous studies that resulted in a change in gene expression often used high doses of TH synthesis inhibiting pharmaceuticals. While the expression patterns of certain genes might be specific to TH synthesis inhibition, it may not be a sensitive endpoint. Future studies focusing on the magnitude of TH synthesis inhibition may be a key to understanding the threshold of inhibition that must occur for TSH independent mRNA transcription to occur. Additionally, the literature currently lacks much information regarding actual levels of environmental and occupational exposure, and it would be useful to know what concentrations of these chemicals are actually being taken up by humans and wildlife. In conclusion, it does not seem that thyroidal mRNA levels of PAX-8, Hex, Tg, NIS, or TPO are useful biomarkers in rats for the indication of short term exposure to TH synthesis inhibitors.

Table 1. Components of dosing mixture. a) Components and highest dose of PHAH mixture. b) Components and highest dose of pesticide mixture.

1a)

Doses and Components of PHAH Mixture

Chemical	Stock Solution A (ug/kg)	50% (ug/kg)
TCDD	0.00390	0.00195
PCDD	0.00390	0.00195
TCDF	0.00570	0.00285
1-PCDF	0.00180	0.0009
4-PCDF	0.00780	0.0039
OCDF	0.0195	0.00975
PCB-28	23.6	11.8
PCB-52	46.6	23.3
PCB-77	0.600	0.3
PCB-101	46.1	23.1
PCB-105	23.0	11.5
PCB-118	114	57.0
PCB-126	0.183	0.0915
PCB-138	114	57.0
PCB-153	115	57.5
PCB-156	3.93	1.97
PCB-169	0.120	0.060
PCB-180	113	56.5
Total	601	301

1b)

Doses and Components of Pesticide Mixture

Chemical	Stock Solution B (mg/kg)	50% (mg/kg)
Pronamide	50.0	25.0
Mancozeb	50.0	25.0
Thiram	75.0	37.5
Total	175	87.5

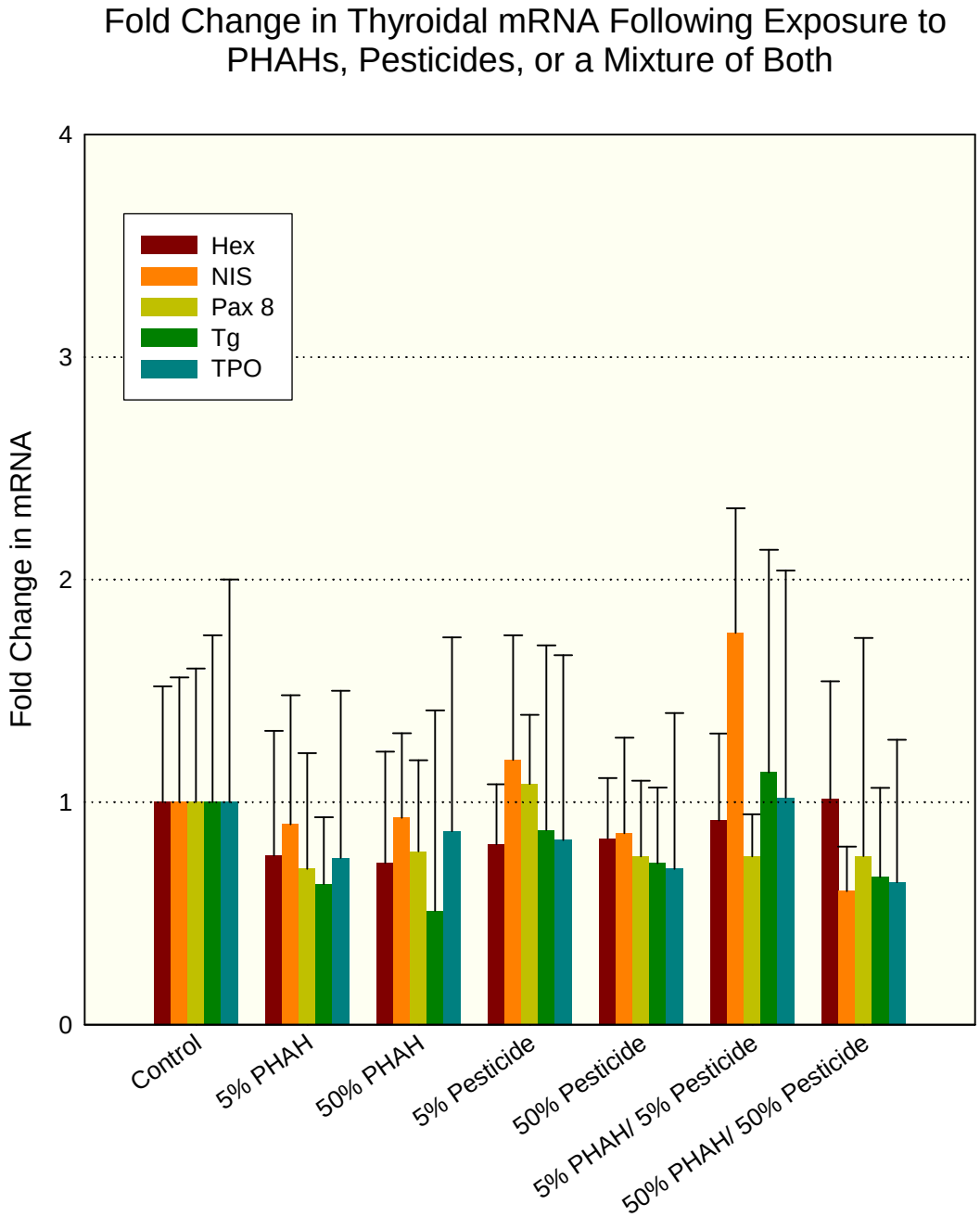


Figure 1. Relative fold change in mRNA (±SD) normalized to β–actin.

CHAPTER IV

Discussion and Conclusions

This research provides further insight into the field of thyroid hormone disruption by demonstrating the effect of a complex mixture on serum hormone concentrations and determining limitations of thyroid mRNA as a biological marker of short term exposure to pesticides. It was hypothesized that decreased circulating T_4 concentrations in rats exposed to a mixture of TH synthesis inhibitors and hepatic enzyme inducers is best predicted by an integrated addition model. Previous work suggested that the effect of PHAHs thought to enhance hormone clearance by upregulating UGTs could be predicted by dose additivity. It was unknown whether a group of chemicals targeting two different components of the hypothalamic-pituitary-thyroid axis would result in dose additive effects on T_4. Comparisons of three model predictions in the present study (dose, effect, and integrated addition) showed that serum T_4 was well predicted by both dose addition and integrated addition. Future studies and mixture modeling techniques will continue to focus on the most appropriate methods to predict biological endpoints.

It was also hypothesized that thyroidal mRNA levels of PAX-8, HEX, Tg, NIS, and/or TPO can be utilized as biomarkers to indicate exposure to chemicals that are TH synthesis inhibitors. Several studies indicated that expression of these genes was TSH dependent, but some *in vitro* studies suggested otherwise. None of the target genes significantly changed following a four day exposure to TH synthesis inhibitors. It appears that either the mechanism of action of thyroid gene transcription differs between *in vitro* and *in vivo* systems and/or changes in these genes are specific to TH synthesis disruption, but not sensitive enough to detect only a partial inhibition of TH synthesis. Therefore, it is not likely that expression profiles of these genes can be used to predict whether exposure to a TH synthesis inhibitor has occurred.

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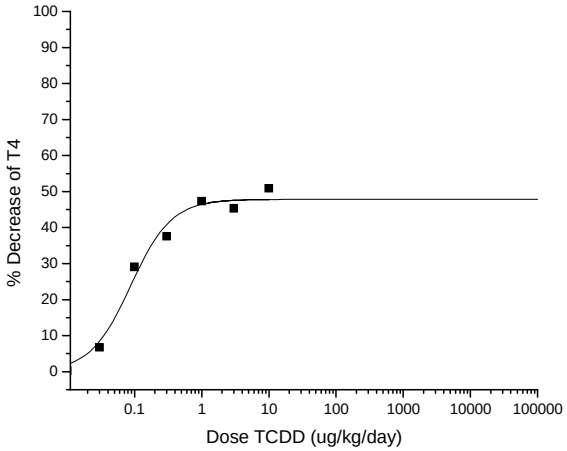
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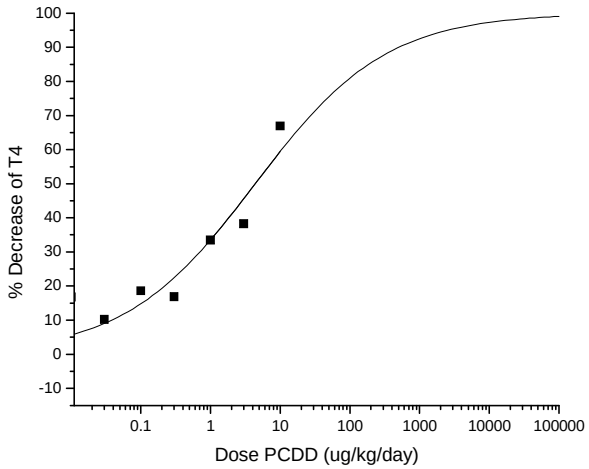
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Appendix: Sigmoidal Fits of Individual Chemicals in the Mixture

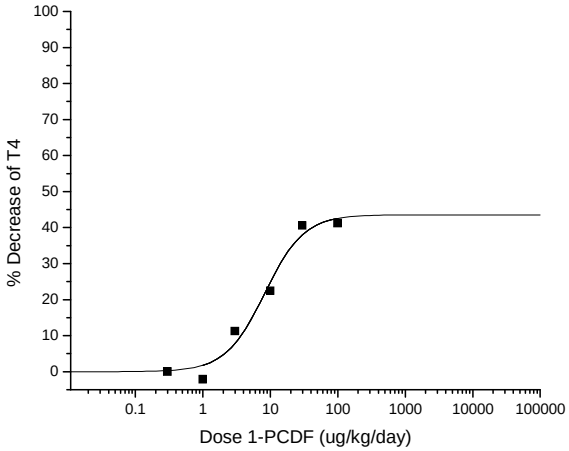
a)


% Decrease of T4
Dose TCDD (ug/kg/day)

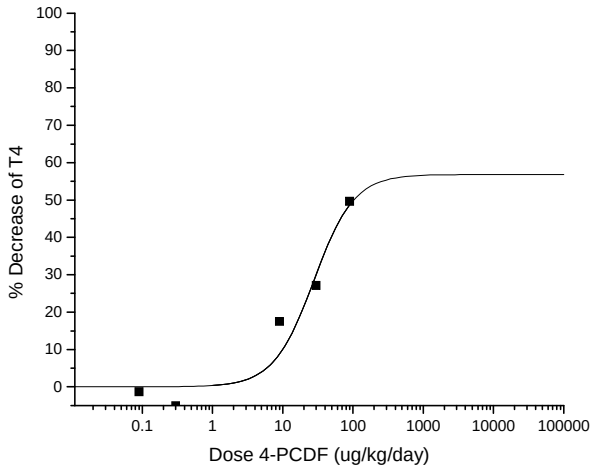
b)


% Decrease of T4
Dose PCDD (ug/kg/day)

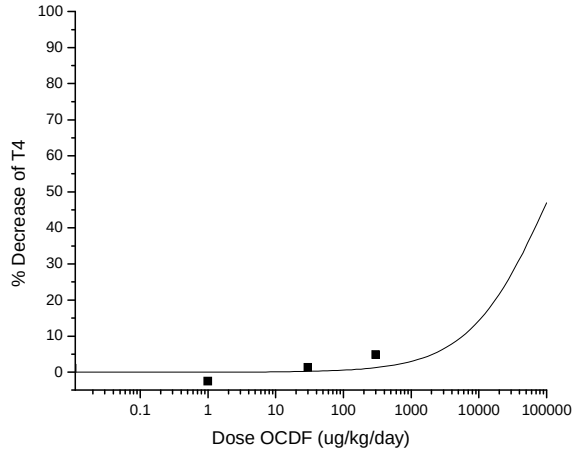
c)


% Decrease of T4
Dose 1-PCDF (ug/kg/day)

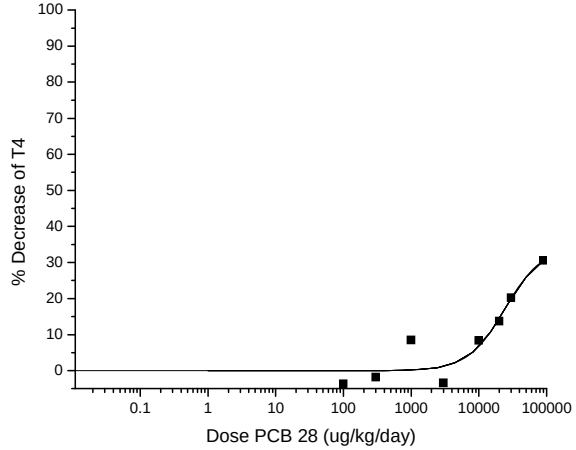
d)


% Decrease of T4
Dose 4-PCDF (ug/kg/day)

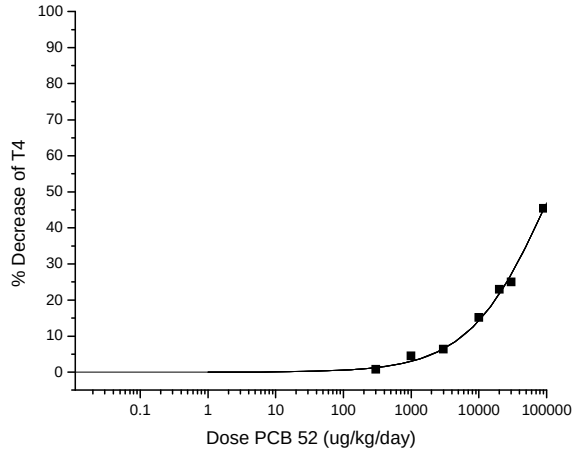
e)


% Decrease of T4
Dose OCDF (ug/kg/day)

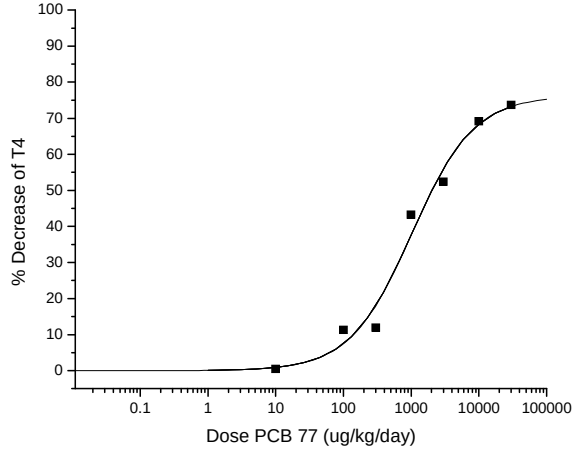
f)


% Decrease of T4
Dose PCB 28 (ug/kg/day)

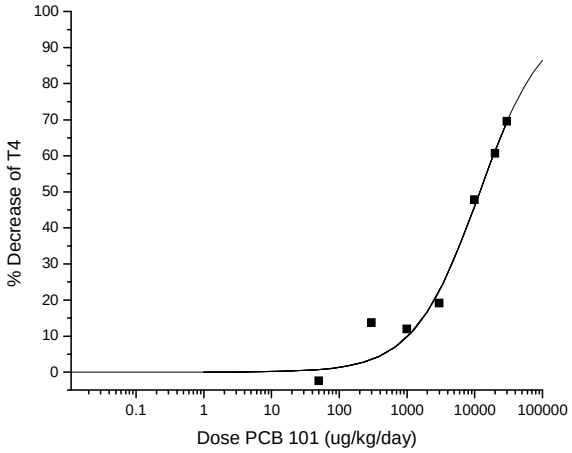
g)


% Decrease of T4
Dose PCB 52 (ug/kg/day)

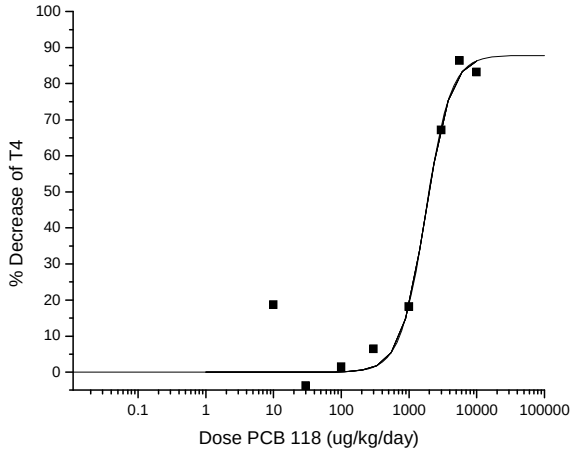
h)


% Decrease of T4
Dose PCB 77 (ug/kg/day)

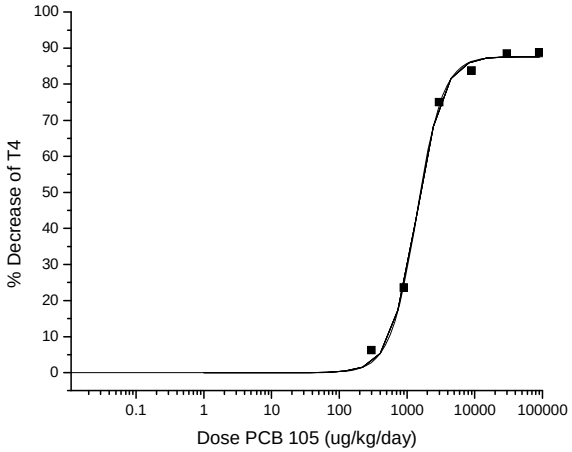
i)


% Decrease of T4
Dose PCB 101 (ug/kg/day)

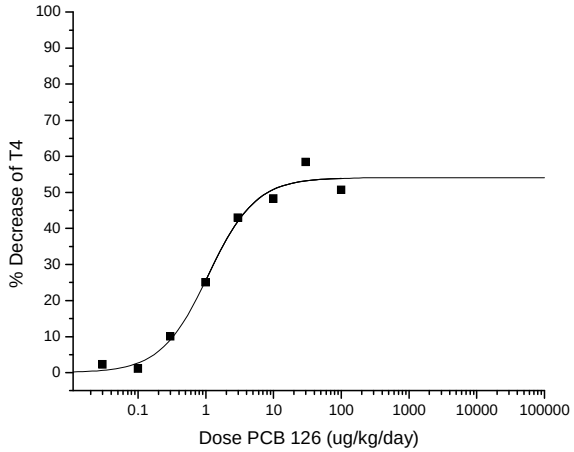
k)


% Decrease of T4
Dose PCB 118 (ug/kg/day)

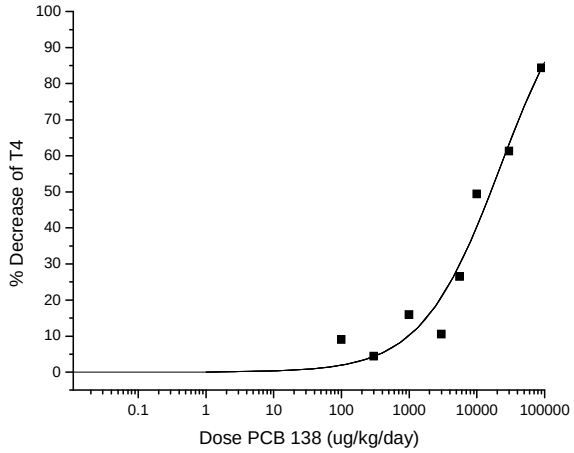
j)


% Decrease of T4
Dose PCB 105 (ug/kg/day)

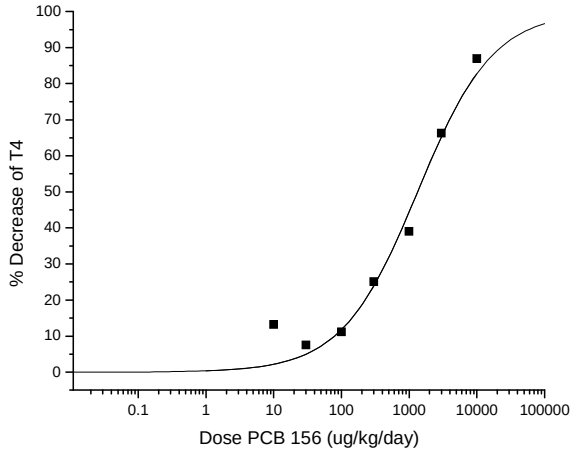
l)


% Decrease of T4
Dose PCB 126 (ug/kg/day)

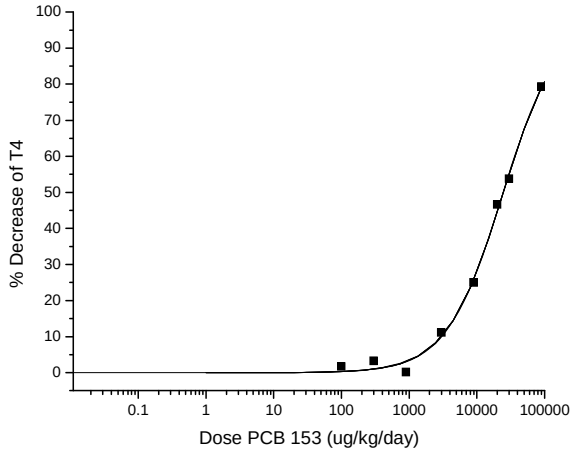
m)


% Decrease of T4
Dose PCB 138 (ug/kg/day)

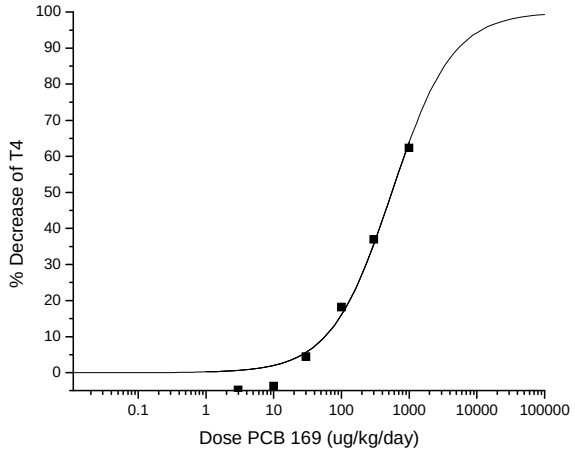
o)


% Decrease of T4
Dose PCB 156 (ug/kg/day)

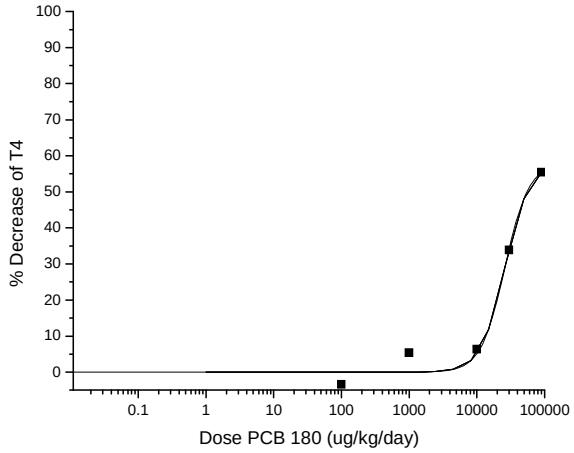
n)


% Decrease of T4
Dose PCB 153 (ug/kg/day)

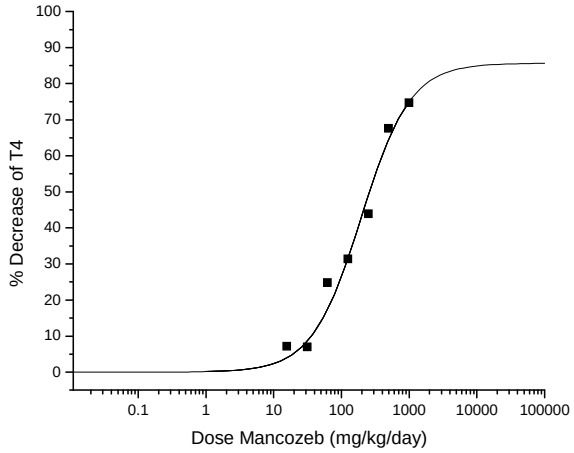
p)


% Decrease of T4
Dose PCB 169 (ug/kg/day)

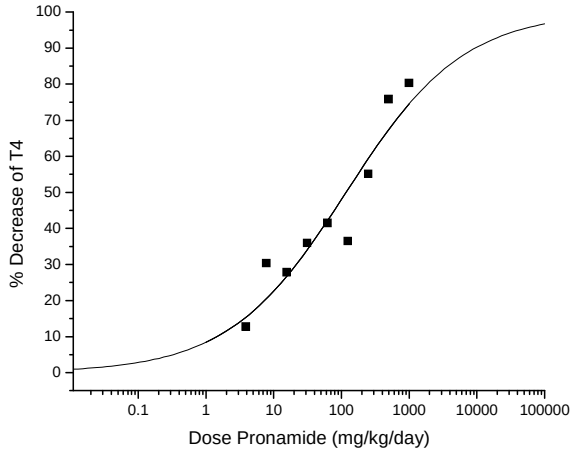
q)


% Decrease of T4
Dose PCB 180 (ug/kg/day)

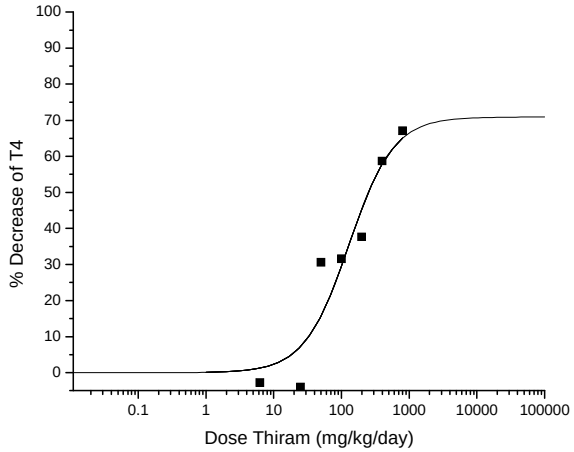
s)


% Decrease of T4
Dose Mancozeb (mg/kg/day)

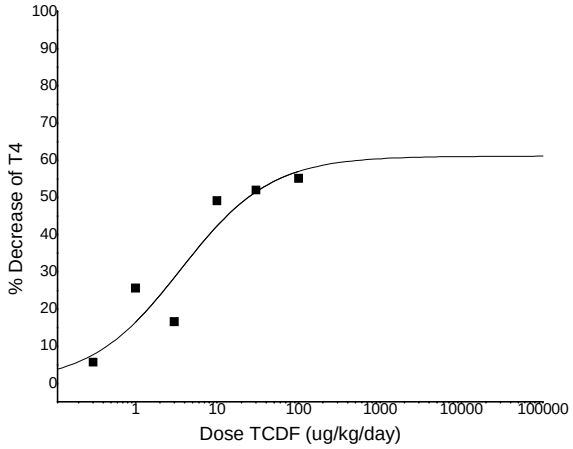
r)


% Decrease of T4
Dose Pronamide (mg/kg/day)

t)


% Decrease of T4
Dose Thiram (mg/kg/day)

u)


% Decrease of T4
100
90
80
70
60
50
40
30
20
10
0
1
10
100
1000
10000
100000
Dose TCDF (ug/kg/day)