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DETAILED REVIEW PAPER ON THYROID HORMONE DISRUPTION ASSAYS

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DETAILED REVIEW PAPER ON THYROID HORMONE DISRUPTION ASSAYS

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FOREWORD

This document presents a Detailed Review Paper on Thyroid Hormone Disruption Assays. At the first meeting of the Validation Management Group for Non Animal Testing (VMG-NA), the International Council on Animal Protection on OECD Programs (ICAPO) presented an initial draft of a Detailed Review Paper (DRP) reviewing in vitro assays for thyroid toxicants. The Endocrine Disrupter Testing and Assessment (EDTA) Task Force that was held in May 2003 agreed that a DRP for "Thyroid Disrupting Assays" should be developed and that it should be a joint effort between the VMG-NA, the VMG for Ecotoxicity Tests (VMG-eco) and the VMG for Mammalian Effects Testing (VMG-mammalian), though led by the VMG-NA. The VMG-NA and VMG-mammalian had both recommended that a DRP be drafted on thyroid mechanisms, both in vitro and in vivo. The EDTA Task Force agreed that this broad overview of test methods for the assessment of the thyroid function was extremely relevant. The United States offered to take the lead taking into consideration the draft paper previously prepared by ICAPO for discussions at the VMG-NA. The Task Force further agreed that the DRP should only focus on methods for the detection of human health hazards and that amphibian assays for the assessment of environmental effects would be outside the scope of the document. A first draft DRP was circulated 31 March 2005 with a deadline for comments on 20 May 2005. Comments were received from Germany, Italy, Japan, the United Kingdom, the United States and ICAPO and were collated by the United States who also edited the document in accordance with the received comments and provided a revised DRP to the Secretariat in March 2006. The Working Group of the National Coordinators of the Test Guidelines Programme approved the DRP at its 18th meeting with some minor changes.

This document is published on the responsibility of the Joint Meeting of the Chemicals Committee and Working Party on Chemicals, Pesticides and Biotechnology.

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1.0 EXECUTIVE SUMMARY

1. This document reviews the state of assays for thyroid toxicants across four vertebrate classes (fish, amphibians, birds and mammals) within the context of a thorough review of thyroid endocrinology across these taxa. Considering the similarity of the endocrinology of the thyroid gland across these taxa, a major feature of this document is the integration of assay comparisons across vertebrates in the final chapter. By assessing the state of the assays amongst mammalian (rodent), fish, amphibian, and avian species, the state of thyroid assays, redundancies, and information gaps presented themselves.

2. Chapter 3 begins a thorough review of the thyroid system in general, introducing the reader to the hypothalamic-pituitary-thyroid axis (HPT axis), in which thyroid hormones T_3 and T_4, are secreted from the thyroid gland under the control of thyroid stimulating hormone (TSH or thyrotropin), which, in turn, is controlled in part by thyrotropin releasing hormone (TRH) from the hypothalamus. Thyroid function is maintained within narrow limits by the negative feedback action of thyroid hormones on both the hypothalamus and pituitary. Recent work indicates that the hypothalamus plays the dominant role in controlling serum T_4 levels. Thyroid hormones are produced in the thyroid gland at the apical border of the thyroid cell (thyrocyte) at the junction of the colloid – a fluid-filled center of the spherical follicles, which are the functional unit of the gland. The hormone "precursor" thyroglobulin is a large protein with many tyrosine residues. These residues are iodinated by the action of thyroperoxidase in association with dual oxidase enzymes that generate H_2O_2 required for the iodination reaction. When the gland is stimulated by TSH, cAMP signalling regulates both an increase in synthesis and secretion of thyroglobulin (Tg). Tg is solubilized by the action of specific Cathepsins in the colloid, then this material is endocytosed into the thyrocyte for transport to the basal surface. During transit through the thyrocyte, the endocytotic vesicle fuses with a lysosome containing Cathepsins, which are enzymes responsible for degrading Tg and liberating T_4 and T_3. Once in the blood, thyroid hormones are carried largely by two proteins – transthyretin (TTR) and thyroxine binding globulin (TBG). In rodents, TTR does not appear to be regulated developmentally or by thyroid hormone; in contrast, TBG levels are high in the neonate but decline in adulthood. In addition, TBG levels are affected by thyroid hormone.

3. Thyroid hormones are cleared from the serum by modifications that make them less lipophilic. These include glucuronidation and sulfation/sulfonation. These alternate pathways of TH metabolism cause their secretion into bile and elimination through the feces. There is some speculation that there is an enterohepatic pathway by which these modified THs can be recycled by the action of enteric bacteria, but there is conflicting evidence in the literature concerning this. Thyroid hormones are taken up into tissues by the action of specific transporters – the organic anion transport protein (OATP) and the monocarboxilic transporter (MCT8). Some cells contain deiodinase enzymes, proteins that can remove an iodine atom from the inner (type 3) or outer (types 1 and 2) ring of the T_4 or T_3 molecule. These enzymes are believed to play an important role in controlling the sensitivity of a cell to thyroid hormone and to respond to changes in thyroid hormone availability in a manner that may compensate for these changes.

4. Thyroid hormone action is mediated by receptors that are nuclear transcription factors. There are two classes of receptors, the TRα and TRβ, encoded by two separate genes. It is possible that the array of receptors is responsible in part for the pleiotropic effects of TH. TH receptors (TRs) bind to specific DNA sequences in enhancer elements of the promoters of specific genes. They are bound to DNA in the unliganded state and act as constitutive repressors. Repression is mediated by the interaction between the TR and a co-repressor. When the TR binds to T_3, the co-repressor is released and a co-activator is recruited. The TRs also mediate the negative feedback action of THs on the hypothalamic-pituitary axis. This system is important to maintain serum TH levels within a narrow range.

5. Thyroid hormones are essential for normal brain development. This is known largely by studying the effect of TH insufficiency. There is very good evidence that the neurological consequences of TH insufficiency is dependent upon the developmental timing of the insufficiency.

6. An important part of Chapter 3 is the description of the many areas of the thyroid system that are not yet well explored as targets for thyroid disruption, for example the thyroid transporters, serum binding proteins, and non-genomic effects on glucose uptake, mitochondria, and actin polymerization and vesicular recycling. Current thyroid research has implications for future assays and the way we measure thyroid disruption. We strongly recommend that the reader review Chapter 3 before considering the information in the rest of the document, as it clearly frames the state of knowledge in this field and the areas where future attention may need to be focused, regardless of one's taxa of interest.

7. The historical background, current literature, and proposed regulatory assays are discussed for 4 animal taxa in chapters 4-7. Chapter 4 focused on mammalian assays, mainly in rodents, and *in vitro* assays based on, but not limited to mammalian systems. Chapter 5 focused on thyroid systems in different fishes and the assays available and current literature available. Chapter 6 discussed amphibian thyroid systems and the assays and endpoints generated to observe effects of toxicants on amphibian development, mainly metamorphosis. Finally chapter 7 focused on the avian thyroid and current assays and literature to describe what is know for birds. A common theme across the chapters is the conserved nature of the thyroid system amongst vertebrate taxa. The structure of T_4 and T_3 is the same in all taxa, as is the mechanism by which they are synthesized. In each taxa reviewed, T_4 is the main hormone produced by the thyroid gland, but T_3 is the active hormone and is converted from T_4 to T_3 in the peripheral tissues. This conversion partially dictates how sensitive different tissues are to thyroid hormones. Thus, blood levels of T_4 represent a measure of thyroid function, and blood levels of T_3 represent a measure of peripheral deiodination of T_4. Because some animals are very small (e.g., amphibian larvae, flounder larvae), it may not always be practical to measure blood levels of hormones. Therefore, it may be necessary to develop and validate methods that utilize tissue for hormone measurements.

8. Each chapter reviews *in vivo* assays available for analysis of thyroid function, and which are being considered for validation by the OECD and US EPA. Assays in rodents that measure the traditional thyroid hormone measures and histopathology are discussed. In addition, assays that are in the development phase, which demonstrate the specific effects of thyroid hormones on rodents (for example, inhibition or delay of cerebellar development), are described. Chapter 4 concludes that the currently utilized endpoints that have been used to identify all known thyroid toxicants to date are the best that are currently available (for example, thyroid hormone measurements, TSH, histopathology, thyroid gland weight). However, there is a need for new endpoints that will determine the specific effects of thyroid toxicants on individuals, but these new endpoints are not yet available for use in the validation of the current generation of assays. Chapter 4 recommends that additional sampling times be added to the current *in vivo* assays for rodents to capture early developmental thyroid hormone measures at PND 5, 15, and 21, and in adult rodents it stresses the importance of capturing early (48 hours) and late (14-28 days) responses of the thyroid system to toxicants.

9. The fish thyroid can be distributed in multiple locations throughout the body, unlike other vertebrates where it is generally found in one location. The fish system also differs slightly in its regulation of thyroid hormone in that it relies more on peripheral regulation of thyroid hormone at the tissue level. The current OECD and US EPA fish assays for screening potential endocrine disruption do not include thyroid endpoints. Potential endpoints or assays that may one day be useful in detecting thyroid disruptors include for example, thyroid hormone measures and the flounder metamorphosis assay.

10. The amphibian thyroid system is described at depth and largely focused on metamorphosis of the frog from tadpole to adult and the well established role of thyroid hormones in this major event. The

amphibian thyroid system is very similar to the mammalian thyroid system in its regulation, structure, and metabolism of thyroid hormones, but during metamorphosis it does not utilize TRH, but rather CRF to regulate TSH. The amphibian metamorphosis assay under validation by the OECD and US EPA is a 21 day assays that begins at stage 51 and observes developmental endpoints combined with thyroid hormone measures and histopathology.

11. Avian thyroid systems also have many similarities to other vertebrate systems, and like the mammals thyroid hormones in the birds have been well linked to growth, brain development and neurogenesis in adults, the skeletal system, thermoregulation, and the reproductive system to name a few. There is no avian screening assay currently in development for use in detecting potential endocrine disruption. There is a comprehensive two-generation test currently being developed by the OECD and US EPA which does include thyroid relevant endpoints for evaluating adverse responses. One endpoint recommended for consideration in birds as well as other vertebrates is the measure of thyroid gland thyroid hormone content, as this was a more sensitive measure than changes in blood thyroid hormone concentrations in Japanese quail following toxicant exposure.

12. Chapter 8 highlights the conserved nature of the thyroid system in its machinery and functions amongst the vertebrates. It is clear that a thyroid active compound may have an effect on multiple organs within a species or even on multiple vertebrate taxa, but these effects may differ due to metabolic and other cellular differences, so it is currently difficult to predict what an effect on one species or organ means for another species or organ. The importance of the major thyroid endpoints utilized in most of the described assays (especially rodent assays), including thyroid histopathology, thyroid weight, TSH and thyroid hormone measures, was emphasized. But a strong call exists for research to further characterize the potential effects of alterations in these endpoints by different toxicant classes, as well as the need for development of better reagents (for example, antibodies for TSH).

13. The complicated nature of the thyroid system, makes development of an *in vitro* battery of assays to detect thyroid disruption unlikely in the near future. This conclusion is based on two facts: the *in vitro* assays available need further development before they can be validated, and the number of *in vitro* assays required to encompass every potential point of disruption in the thyroid system would be too great for a manageable assay battery. Furthermore, *in vitro* assays alone would not detect altered interactions within the thyroid system in response to toxicants. However, recommendations were made on *in vitro* assays that could be developed and utilized for high throughput screens in the future. Chapter 8 also points out that the recommended *in vivo* endpoints for future development would likely serve as add-ons to the existing assays and would not increase the number of animals utilized in an assay battery.

14. The review document does not recommend a strategy to test for thyroid disruption, but rather identifies what is currently ready for validation or in need of more research and development to improve the currently proposed assays. The purpose of the document was not to suggest one battery to test for thyroid disruption, but to provide the scientific and regulatory community with the information that will help them to make choices on the assays they will need for their individual purposes. The authors of this document hope that it will serve its intended purpose and generate productive discussion, useful research, and effective testing strategies.

2.0 INTRODUCTION

2.1 History and Genesis of a Comprehensive Review of Thyroid Assays across Taxa

15. The Organization for Economic Cooperation and Development (OECD) initiated a high-priority activity in 1997 to develop new test guidelines and revise existing test guidelines for the screening and testing of potential endocrine disruptors. This activity is organized under the Task Force on Endocrine Disruptors Testing and Assessment as part of the OECD test guidelines program and managed by three Validation Management Groups (VMGs) covering mammalian, ecotoxicity, and non-animal methods. At the first meeting of the VMG non-animal, the International Council on Animal Protection in OECD Programs (ICAPO) presented an initial draft of a Detailed Review Paper (DRP) reviewing *in vitro* assays for thyroid toxicants. In 2003, all three VMGs identified thyroid screening and testing as areas for further investigation by the OECD. In response to this concern and a request by ICAPO for assistance in completing the DRP on *in vitro* thyroid screening methods, the U.S. Environmental Protection Agency (EPA) agreed to sponsor the preparation of this comprehensive DRP on thyroid toxicity screening and testing methods.

16. The US EPA established the Endocrine Disruptor Screening Program (EDSP) as a result of the passage of the Food Quality Protection Act (FQPA) and an amendment to the Safe Water Drinking Act (SDWA) in 1996. The EPA relied on an Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) to develop a proposed battery of assays for screening and testing that could be used to determine if chemical compounds disrupt the endocrine system. EDSTAC specifically focused their efforts on assays to detect estrogen, androgen, and thyroid system disruption.

2.2 Purpose of the Present Review

17. The present document is designed to review several major research areas that are important to those engaged in programs focused on detecting thyroid disrupting compounds and understanding their potential adverse effects. First, a major goal of this DRP is to provide a thorough and up-to-date review of the biology and toxicology of thyroid endocrinology among vertebrates. Second, it was designed as a comprehensive analysis of the strengths and weaknesses of present and proposed assays designed to identify thyroid toxicants. Thus, the specific purposes of this review are to: 1) review the *in vitro* thyroid assays that are capable of detecting interference with the thyroid system; 2) discuss the performance of the *in vitro* assays, in the context of the established *in vivo* assays, for their ability to detect thyroid-related effects in both screening and testing tiers; 3) perform a comprehensive species comparison and generate discussion on the concept that one vertebrate assay may produce generalized information about the ability of a chemical to affect other vertebrate thyroid systems (for example, does a fish- or frog-based *in vitro* or *in vivo* assay indicate thyroid system abnormalities in all vertebrates? Could it serve as a Tier 1 assay that is sufficient for indicating a potential response in any Tier 2 assay for thyroid?); 4) outline new research that may be informative to detecting thyroid disruption, and determine whether alternative screens or tests exist for thyroid that can be more easily linked to adverse consequences than the present tests; and 5) identify strategies to reduce animal use.

2.3 Objective of the Different Assays

18. A number of the screens and tests discussed in this document are intended to inform the needs of the OECD parties that are interested in this document. Thus, it is not the intention of this review to provide examples of batteries of screens or tests that could be employed within the context of the US EPA's Endocrine Disruptor Screening Program (EDSP) or the OECD's Endocrine Disruptor Task Force. Rather, a large number of screens and tests are described for all vertebrates (except reptiles) that can be assembled

into more than one integrated battery to accomplish the goals of chemical identification (depending on the user) as well as to minimize cost and animal usage.

2.4 Methodology used in the Analysis

19. Each chapter was written by an expert in that field, and the first draft was reviewed by outside experts via the OECD. The literature cited in this document was gathered by a large number of broad and focused electronic literature searches of national databases (MEDLINE, TOXLINE). Specific screens and tests described in this document represent existing assays for thyroid toxicity or represent the respective authors' original concepts of effective assays.

2.5 Definitions

20.

Abbreviation	Definition
AVT	Arginine vasotocin
BTEB	Basic transcription element binding protein
CBP	Cortisol binding protein
CRF	Corticotrophin releasing factor
CTHBP	Cytoplasm TH binding proteins
D1	Type I deiodinase
D2	Type II deiodinase
D3	Type III deiodinase
EDSP	Endocrine Disruptor Screening Program
EPA	Environmental Protection Agency
FSH	Follicle stimulating hormone
GHRH	Growth hormone releasing hormone
GnRF	Gonadotropin releasing factor
IRD	Inner-ring deiodinases
LH	Luteinizing hormone
OECD	Organisation for Economic Co-Operation and Development
ORD	Outer-ring deiodinases
PVN	Para ventricular nucleus
RPA	Ribonuclease protection assay
RT-PCR	Real-time Polymerase Chain Reaction
SS	Somatostatin
T_3	Triiodothyronine
T_4	Thyroxin
TBG	Thyroxin-binding globulin
Tg	Thyroglobulin
TH	Thyroid hormone
TRH	Thyrotropin-releasing hormone
TSH	Thyroid stimulating hormone (thyrotropin)
TTR	Transthyretin
VMG	Validation Management Group

3.0 GENERAL BACKGROUND ON THE HYPOTHALMIC-PITUITARY-THYROID (HPT) AXIS

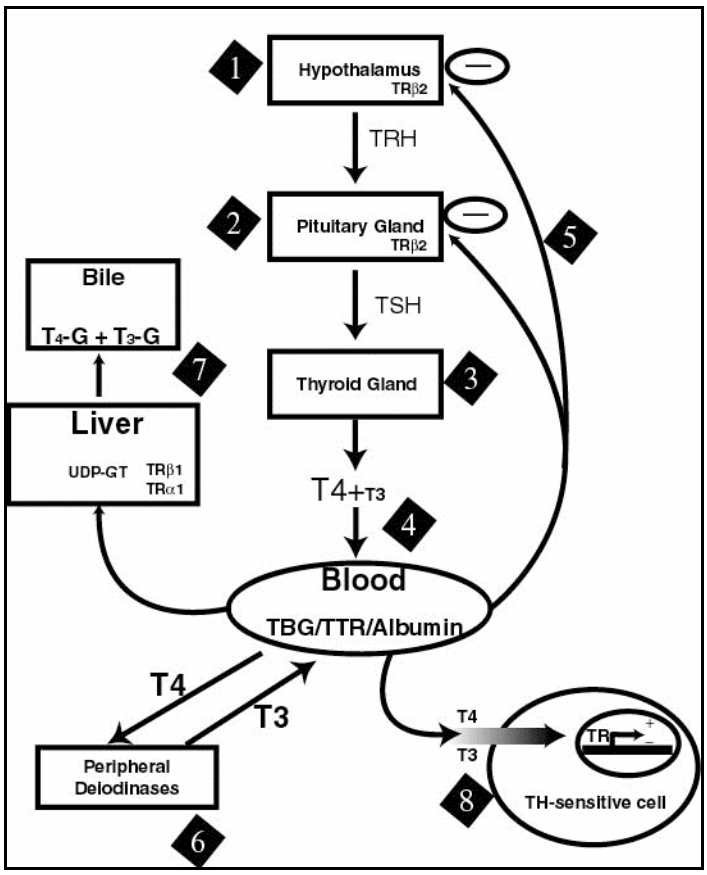
21. Thyroid hormone is essential for normal development, and for maintenance of normal physiological functions. The regulation of thyroid hormone delivery to tissues and cells during development and in the adult represents a very complex and unique (among endocrine systems) web of feedback systems providing redundant and compensatory regulatory responses to maintain thyroid hormone signaling in the face of specific deficiencies in circulating levels of thyroid hormones. Environmental factors, such as iodine deficiency or the presence of specific toxicants, can perturb this web at various points of regulation, inducing a variety of responses that are captured in toxicological assays. However, more research is needed to truly understand the degree of thyroid disruption induced by a particular toxicant that is necessary to be considered either "compensatory/adaptive" or "adverse" with respect to non-cancer endpoints. Changes in thyroid hormone levels due to chemical exposure are generally considered adverse, but the thyroid field is still new and continued research on new endpoints of thyroid hormone action is needed to link the changes in thyroid hormone levels to dose of chemical and clear downstream actions. There is good evidence that the mechanisms by which different toxicants disrupt the thyroid system determine the degree to which adverse effects can be inferred. Clearly, to assemble a battery of screens and tests that will generate the information required to identify thyroid toxicants that may disrupt the thyroid system, assays must be developed based on what is known about thyroid endocrinology. Therefore, the goal of this section is to provide a basic primer to the thyroid endocrine system to explicitly address the endocrinology of the thyroid system and the relevant differences between rodents and humans that will affect the informed design of screens and tests for thyroid toxicants. In addition, comparisons among other taxa will be briefly highlighted within this chapter, but expanded in the following chapters.

22. We begin by providing an outline of the hypothalamic-pituitary-thyroid (HPT) axis, with brief descriptions of the various functional levels of the axis. Following this description, we present detailed background information with references to the points of thyroid disruption known to occur.

3.1 The Hypothalamic-Pituitary-Thyroid (HPT) Axis

23. Thyroid toxicants are generally defined as toxicants that alter circulating levels of thyroid hormone (Brucker-Davis 1998). However, studies of thyroid disruption often incorporate an incomplete picture of the dynamic relationships within the mammalian HPT axis, and for good reason. These relationships are quite complex, and measurement of some of these parameters (e.g., free hormone level in fetal serum) can be very difficult to obtain (see Figure 3-1). Moreover, toxicants interfering with the HPT axis by different mechanisms appear to produce different effects on the relationship among the various endpoints within the thyroid endocrine system. Thus, it is important to capture endpoints that are more uniformly indicative of thyroid disruption as well as reflective of adverse effects. Therefore, we first present an overview of the HPT axis as developed in mammals, followed by a more detailed description of each of the components of this axis. Finally, we provide a review of feedback relationships among the levels of the HPT axis. In all cases, the focus is on mammalian thyroid endocrinology, with mention of comparative thyroid endocrinology throughout this review.

Figure 3-1 The Mammalian Hypothalamic-Pituitary-Thyroid Axis



Numbers in filled diamonds refer to the legend below that provides descriptions

1. Neurons whose cell bodies reside in the hypothalamic paraventricular nucleus (PVN) synthesize the tripeptide Thyrotropin-Releasing Hormone (TRH) (Segersen et al. 1987a; Segersen et al. 1987b). Although TRH-containing neurons are widely distributed throughout the brain (Jackson et al. 1985; Lechan et al. 1986), TRH neurons in the PVN project uniformly to the median eminence (Ishikawa et al. 1988; Merchenthaler and Liposits 1994), a neurohemal organ connected to the anterior pituitary gland by the hypothalamic-pituitary-portal vessels (Martin and Reichlin 1987), and are the only TRH neurons to regulate the pituitary-thyroid axis (Aizawa and Greer 1981; Taylor et al. 1990b).

2. TRH is delivered by the pituitary-portal vasculature to the anterior pituitary gland to stimulate the synthesis and release of Thyroid Stimulating Hormone (TSH) or "Thyrotropin" (Haisenleder et al. 1992). TRH selectively stimulates the synthesis of the TSH beta subunit (Haisenleder et al. 1992). However, TRH also affects the post-translational glycosylation of TSH which affects its biological activity (Taylor and Weintraub 1985; Lippman et al. 1986; Taylor et al. 1986; Weintraub et al. 1989; Magner et al. 1992; Harel et al. 1993). To our knowledge, there is no empirical evidence that TSH exerts a negative feedback effect on TRH neurons of the PVN. Zoeller et al. did not find that TSH affected TRH neurons of the PVN, nor did it affect the ability of thyroid hormone to influence these neurons (Zoeller et al. 1988). Interestingly, a recent report by Nikrodhanond et al. (2006) demonstrates that the role of TRH in regulating the pituitary-thyroid axis is stronger than the role of TH negative feedback.

3. Pituitary TSH is one of three glycoprotein hormones of the pituitary gland and is composed of an alpha and a beta subunit (Wondisford et al. 1996b). All three pituitary glycoproteins (Luteinizing Hormone, LH; Follicle Stimulating Hormone, FSH; and TSH) share the same alpha subunit (Hadley 2000). Pituitary TSH binds to receptors on the surface of thyroid follicle cells stimulating adenylate cyclase (Taurog et al. 1996; Wondisford et al. 1996a). The effect of increased cAMP is to increase the uptake of iodide into thyroid cells, iodination of tyrosyl residues on thyroglobulin (TG) by thyroperoxidase, synthesis and oxidation of TG, TG uptake from thyroid colloid, and production of the iodothyronines T_4 and T_3. T_4 is by far the major product released from the thyroid gland (Taurog et al. 1996). Recent anatomical studies have shown that human pituitary thyrotropes express the mRNA encoding the TSH receptor (Prummel et al. 2000; Theodoropoulou et al. 2000), which may represent a negative feedback loop accounting for the fact that serum TSH is reduced in some Graves' patients with normal levels of thyroid hormone (Prummel et al. 2004).

4. Thyroid hormones are carried in the blood by specific proteins. In humans, about 75% of T_4 is bound to thyroxine-binding globulin (TBG), 15% is bound to transthyretin (TTR) and the remainder is bound to albumin (Schussler 2000). TBG, the least abundant but most avid T_4 binder, is a member of a class of proteins that includes Cortisol Binding Protein (CBP) and is cleaved by serine proteases in serum (Fink et al. 1986). These enzymes are secreted into blood during inflammatory responses and, in the case of CBP, can induce the release of cortisol at the site of inflammation. The physiological significance of this observation is presently unclear for TBG (Schussler 2000). However, it is clear that T_4 is released from TBG upon cleavage (Grasberger et al. 2002; Janssen et al. 2002). The presence and abundance of the different binding proteins varies among the vertebrates and may be developmentally regulated in a generalized manner. In the rat, high serum levels of TBG are found in the fetus and the early postnatal pup (Vranckx et al. 1990, 1994); adult levels of TBG are undetectable, but low serum T_4 appears to increase both serum TBG and liver biosynthesis in the rodent (Vranckx et al. 1994). Interestingly, organisms across taxa appear to have the greatest carrying capacity for T_4 in serum during development compared to their respective adult forms (Richardson 2005). This may be a mechanism by which T_4 can be adequately maintained at a developmental time when it is uniformly important.

5. Thyroid hormones (T_4 and T_3) exert a negative feedback effect on the release of pituitary TSH and on the activity of hypothalamic TRH neurons (Koller et al. 1987; Segersen et al. 1987b; Rondeel et al. 1989). Although it is clear that thyroid hormone regulates the expression of TSH (Franklyn et al. 1987; Mirell et al. 1987; Shupnik and Ridgway 1987) and TRH (Koller et al. 1987; Segersen et al. 1987a; Segersen et al. 1987b; Zoeller et al. 1988) in a negative feedback manner, it is also clear that the functional characteristics of negative feedback must include more than simply the regulation of the gene encoding the secreted protein/peptide. In addition, fasting suppresses the activity of TRH neurons by a neural mechanism that may involve leptin (Lagradi et al. 1997; Fekete et al. 2000). This fasting-induced suppression of TRH neurons results in the reduction of circulating levels of thyroid hormone. In humans and perhaps in rodents, circulating levels of T_4 and of T_3 fluctuate considerably within an individual; therefore, TSH measurements are considered to be diagnostic of thyroid dysfunction (Roti et al. 1993; Chopra 1996; Stockigt 2000). However, individual T_4 levels in humans vary within far narrower limits than the population limits (i.e., the population reference range) (Andersen 2002, 2003). In addition, variance in serum T_4 in pairs of monozygotic twins is far more correlated than that in pairs of dizygotic twins or the general population (Hansen 2004). Thus, the set-point around which negative feedback appears to function has a very strong genetic component in humans and perhaps in other animals.

6. T_4 and T_3 are actively transported into target tissues (Oppenheimer 1983; Everts et al. 1994a; Everts et al. 1994b; Everts et al. 1995; Kragie 1996; Docter et al. 1997; Friesema et al. 1999; Moreau et al. 1999). T_4 can be converted to T_3 by the action of outer-ring deiodinases (ORD, Type I and Type II) (St Germain and Galton 1997). Peripheral conversion of T_4 to T_3 by these ORDs accounts for nearly 80% of the T_3 found in the circulation (Chopra 1996).

7. Thyroid hormones are cleared from the blood in the liver following sulfation or sulfonation by sulfotransferases, or following glucuronidation by UDP-glucuronosyl transferase (Hood and Klaassen 2000a, 2000b). These modified thyroid hormones are then eliminated through the bile.

8. T_4 and/or T_3 are actively concentrated in target cells about 10-fold over that of the circulation, although this is tissue-dependent. The receptors for T_4 and T_3 (TRs) are nuclear proteins that bind to DNA and regulate transcription (Lazar 1993, 1994; Oppenheimer et al. 1994; Mangelsdorf and Evans 1995; Oppenheimer and Schwartz 1997). There are two genes that encode the TRs, c-erbA-alpha (TRα) and c-erbA-beta (TRβ). Each of these genes is differentially spliced, forming 3 separate TRs, TRα1, TRβ1, and TRβ2. The effects of thyroid hormone are quite tissue-, cell-, and developmental stage-specific and it is believed that the relative abundance of the different TRs in a specific cell may contribute to this selective action.

3.2 Structure of the Mature Thyroid Gland

24. The mature human and rodent thyroid gland consists of two elongated oval lobes, one on each side of the trachea, joined near their posterior poles by a thin isthmus crossing the trachea ventrally (see (Braverman and Utiger 2004). The lobes, buried under the muscles of the neck region, are richly vascularized and made up of groups of fluid-filled spheres, or follicles, often visible macroscopically. The lobes extend anteriorly as far as the cricoid cartilage of the larynx and posteriorly over the first three or four tracheal rings. Variations in size, extent, and/or position are common. In fact, the specific overall shape of the gland differs among some mammalian species, though the microscopic structure remains the same. Similarly, the location and gross structure of thyroid gland tissue among all jawed-vertebrate species may differ, but the microscopic structure remains the same.

25. Microscopically, the gland is made up of fluid-filled follicles of varying sizes, surrounded by a fibrous connective tissue capsule and supported by richly vascular interfollicular connective tissues. Simple cuboidal cells with distinct outlines, large spherical nuclei, and clear cytoplasm line the follicles. The height of the epithelial cells and the amount and staining quality of the colloid are generally believed to be indicative of secretory activity.

26. Thyroid follicles are relatively uniform in size, though they appear variable in size in histological sections because of the different amounts of follicles contained within thin sections. They contain a homogeneous, slightly acidophilic colloid. During the early postnatal months (1 to 3 months in strain C3H mice and 1 to 5 months in strain C57 mice), cuboidal cell height decreases rapidly and follicle diameter increases rapidly. With increasing age, follicles become larger, the interfollicular tissue decreases, and the colloid becomes more eosinophilic. Senile changes, which occur as early as 12 months of age in mice of some strains and more markedly in females, include loss of stainable colloid, increase in fibrous interfollicular connective tissue, and great variation in follicle size. It is not uncommon to observe coalescence of contiguous large follicles to form bilocular or trilocular cysts with flattened epithelium. These observations demonstrate that there are developmentally associated dynamic changes in the histological structure of the mammalian thyroid gland, and thus, experiments must be timed to ensure that normal variation in structure does not confound the findings.

27. In general, the thyroid gland is structurally conservative among jawed vertebrates (Gnathostomes). The gland is often a collection of aggregated follicles as described for mammals, highly vascularized and encapsulated by connective tissue. Especially in fish, these follicles can be distributed diffusely in the pharyngeal region. These organizational differences impact studies because histopathology is much more difficult in fishes than in mammals, considering the diffuse organization. However, despite these structural differences, the biochemistry and regulation of thyroid hormone synthesis is identical.

3.3 Development of the Thyroid Gland

28. The paired ultimobranchial bodies (sometimes termed the lateral thyroid) are thought to form from the rudimentary fifth pharyngeal pouches beginning in the human at five to six weeks *in utero*. At the beginning of the seventh week (13 mm embryo), each ultimobranchial body, joined with the adjacent pair of parathyroids (from the fourth branchial pouches), separates from the pharynx and comes in contact with the growing two lobes of the thyroid. The ultimobranchial bodies lose their lumina and become incorporated into the thyroid gland. The ultimate fate of these bodies is not certain. They may degenerate, be converted (induced) to differentiate into thyroid tissue, form physiologically and morphologically distinct follicles within the thyroid, or ultimately form the parafollicular (calcitonin) cells of the thyroid.

29. The thyroid gland, *per se*, is the first glandular structure to form. Even the human embryo at three weeks of age, of six somites (2 mm long), exhibits an external bulge on the ventral floor of the foregut, just caudal to the pharyngeal membrane and cephalad to the pericardial cavity. A distinct endodermal outpocketing, the thyroid diverticulum, soon protrudes (by the time the embryo is 4.5 mm) and lies between the second pair of pharyngeal pouches (see above). This sac initially maintains its connection to the pharynx by a narrow neck termed the thyroid glossal duct (first observed at 8 mm), so named because it is initially hollow and connects the primitive thyroid with the tongue, which is forming from the pharyngeal floor at the same time (about four weeks *in utero*). The duct opens at the aboral end of the median swelling of the tongue (the tuberculum impar). The duct becomes a solid stalk and disintegrates in the sixth week *in utero*, but its point of origin on the tongue is permanently marked by an enlarged pit termed the foramen caecum.

30. The thyroid sac quickly becomes a solid bilobed mass that lies against the primitive aortic stem. When the stalk atrophies, the thyroid converts to an irregular mass of epithelial plates. Early in the seventh week *in utero* in humans, the gland becomes C-shaped and settles into a transverse position with a lobe on each side of the trachea. The transverse position is caused by the forward growth of the pharynx, which leaves the aortic trunk and thyroid gland below it. Also during the seventh week, the enlarging ultimobranchial bodies come in contact with the thyroid primordium and fuse with it (see above), thereby separating the thyroid from the aorta and pericardium. In the eighth week *in utero* in humans, discontinuous cavities begin to appear in swollen or beaded portions of the solid thyroid plates. These cavities are the beginnings of the follicles that acquire colloid in the third month *in utero* and soon after become functional. By the end of the fourth month *in utero*, this conversion into follicles ceases. Thereafter, new follicles form only by the budding and subdivision of those already present. A capsulated vascular stroma differentiates from the local mesenchyme.

31. This same thyrogenic process occurs in similar (or identical) fashion in all mammals. In the fetal pig, the pharyngeal pouches and thyroid gland form similarly with the gland located between the second and third branchial arches, with the thyroglossal duct initially opened just caudal to the tubercular impar (unpaired median swelling) of the tongue. In the mouse, the thyroid gland also forms from a medial epithelial mass growing ventrally at the level of the first and second pharyngeal pouches. The ultimobranchial bodies from pouches IV and V become closely integrated with the median thyroid mass and may form structures that persist in the adult thyroid. Some of these ultimobranchial body-derived follicular tissues are physiologically and morphologically distinguished from the medullary-derived

thyroid; ultimobranchial-derived follicles (at least in mice) have ciliated epithelial cells. These follicles with ciliated cells are particularly conspicuous in strain C3H mice, where they have been observed in newborns. In mice, thyroid function is initiated in 15- to 17-day-old fetuses *in utero*, with colloid secretion preceding follicle formation. Even in the chick embryo (Class Aves), the pharyngeal pouches and branchial grooves (only three, not five) form similarly. In this class, the thyroid gland forms at the level of the second pair of arches from the median floor of the pharynx.

32. Despite these differences in development and adult anatomy of the thyroid gland among the vertebrate taxa, several morphological, chemical, and functional commonalities exist. For example, thyroid hormones (T_4 and T_3) are chemically identical in all vertebrates. Moreover, these molecules are synthesized as part of a large protein (thyroglobulin). Because thyroglobulin is iodinated at the interface of the thyroid follicle cell and the colloid, all thyroid hormone producing tissues in vertebrates must form follicles. However, the organization of follicles into discreet glands differs among the vertebrates as described above. In addition, thyroid function is regulated by TSH in all vertebrates, and this pituitary hormone is regulated by a combination of negative feedback effects of thyroid hormone and by the stimulatory effects of the hypothalamus. However, the tripeptide TRH, which controls pituitary TSH release in mammals and birds, does not appear to control pituitary TSH release in amphibians. Finally, equivalent molecules in all vertebrate taxa control thyroid hormone action. Specifically, all vertebrates express thyroid hormone receptors, and these receptors regulate gene expression. The details of these events are described more fully in the following chapters.

3.4 Structure of the Mature Parathyroid Glands

33. The parathyroid glands produced parathyroid hormone (PTH), and though these glands are regulated independently of the thyroid and the hypothalamic-pituitary-thyroid axis, studies involving thyroidectomy often must deal with the confounding variable of the lack of PTH. In the human (and other mammalian) embryo, the parathyroid glands form from the dorsal portions of the third and fourth pairs of pharyngeal pouches; the third pair forms the adult inferior parathyroids, and the fourth pair remains at the cranial thyroid border and forms the superior parathyroids. In the adult, the position and number of parathyroid lobes are variable, although usually in mice a single lobe lies just under the capside near the dorolateral border of each lobe of the thyroid. Two members of a pair are seldom at the same anteroposterior level. Sometimes one or both may be posterior to the thyroid; they may be deeply embedded in the thyroid tissue, and/or there may be more than two parathyroid lobes.

34. Each parathyroid gland in the mouse is usually separated from the thyroid by a connective tissue capsule and consists of sheet-like masses and anastomosing cords of polygonal cells separated by a network of capillaries or sinusoids. Specific cell types are identified with their relative abundance varying with age: (1) the principal cells have large vesicular nuclei and scanty basophilic cytoplasm; (2) ovoid to fusiform-shaped cells with smaller hyperchromatic nuclei and more abundant granular eosinophilic cytoplasm in small groups in the interstitial connective tissue (these increase with age); (3) very large cells with large vesicular nuclei and prominent nucleoli (these become conspicuous only in old age); and (4) pigmented dendritic cells in the parathyroid stroma of pigmented mice (most frequently in strain C58 mice). Because the parathyroids develop in close proximity to the developing thymus, ultimobranchial bodies, and thyroid, they may remain in contact with these organs in adulthood. Parathyroid “nests” (distinguishable histochemically) have been consistently found in the thymus septa or surface connective tissue, and sometimes the parathyroid, thyroid, and thymus are found connected by a ciliated cyst.

3.5 Overview of Functional Relationships among Levels of the Hypothalamic-Pituitary-Thyroid Axis

35. Current screens and tests for thyroid toxicants are based on the interactions among hormones within the hypothalamic-pituitary-thyroid (HPT) axis. These interactions include trophic actions (i.e., stimulatory effects) and inhibitory effects (i.e., negative feedback). Therefore, this section was developed to provide the background information required to understand the current assays, and additional information to develop new assays to identify endocrine toxicants. This overview is generated largely from experimental work in rodents (mostly rats and mice). However, some information is provided to demonstrate the similarity with the HPT axis in humans where this information is available. We have attempted to clarify the origin of the information throughout.

3.5.1 The Hypothalamic-Pituitary-Thyroid Axis

36. The thyroid gland is controlled principally by an interaction between iodine availability, a requirement for thyroid hormone synthesis, and thyrotropin (TSH) from the pituitary gland. This is true for humans, for rodents (Morreale de Escobar et al. 1997; Dunn and Dunn 2000), and in other vertebrates (Norris 1996). In turn, TSH, a glycoprotein hormone, is under the regulation of thyroid hormone itself (negative feedback) and of the releasing factor, thyrotropin releasing hormone (TRH) from the hypothalamus. TRH release is controlled by thyroid hormone (the long loop of the negative feedback system) as well as by neural inputs that relay information about a variety of physiological states including food availability, body temperature, and perhaps cardiovascular functioning. Greer et al. (1993) proposed that TRH controls the set point around which thyroid hormone regulates TSH release, suggesting that TSH regulation is a pivotal point of regulation within the HPT axis.

37. Although these are the central features regulating thyroid function within the HPT axis, there are many additional processes that contribute to the overall regulation of the HPT axis, and of thyroid hormone action at target tissues. Perhaps most important among these are the metabolic enzymes that control changes in the iodination state of thyroid hormone. Specifically, three classes of deiodinase enzymes control the conversion of thyroid hormone to various active and inactive forms. It is becoming clear that the activity of these enzymes can contribute significantly to regulating tissue sensitivity to thyroid hormone and their enzymatic activity can be affected by various toxicants. In addition, enzymes in the liver target thyroid hormone for covalent modifications that lead to removal of thyroid hormones from the circulation. Likewise, the enzymes induced by some toxicants may produce significant changes in serum hormone levels as a result. Finally, serum binding proteins are important in regulating total hormone levels by increasing the carrying capacity of iodothyronines, which are only poorly soluble in aqueous media. Serum binding proteins are themselves regulated by a variety of factors, including thyroid hormone; thus, chemicals that change circulating levels of thyroid hormones are also likely to alter serum binding proteins which will further change (in an adaptive manner or not) the dynamics of this endocrine system. Each of these steps is reviewed in detail below.

3.5.2 Thyroid Hormone Synthesis

38. Thyroid hormone is synthesized in a very different way than other hormones that use similar signaling pathways (i.e., steroid hormones). Figure 3-2 illustrates the structure and function of the thyroid. Thyroid hormone is a small bi-phenolic compound derived from separate tyrosine residues on a large protein – thyroglobulin (Tg) (Taurog 2004). Thyroglobulin, in turn, is synthesized on ribosomes and transported (by exocytosis) to the colloid. It is then iodinated at specific tyrosine residues *as it is being exocytosed*. This large iodinated protein—thyroglobulin—is then stored in the colloid until it is required for the synthesis of hormone. At that time, colloid droplets are ingested by thyroid follicle cells by endocytosis and transported to the side of the cell that is bathed in interstitial fluid. On the way through the

cell, the endocytotic vesicle fuses with a lysosome. Enzymes within the lysosome then digest the iodinated Tg and liberate T_4 and T_3. These steps are expanded below.

3.5.2.1 Regulation of Synthesis by TSH

39. Thyrotropin ("thyroid stimulating hormone" or TSH) regulates the activity of the thyroid gland, including synthesis and release of thyroid hormones, uptake of iodine, and even cell hypertrophy and hyperplasia (Spaulding 2000). When TSH binds to its receptor on the thyroid cell, the intracellular domains of the receptor activate several guanine nucleotide-binding (G) proteins (Wonerow et al. 2001). Cyclic AMP-dependent protein kinases (PKA) mediate many of the actions of the activated TSH receptor including its mitogenic action (Dremier et al. 2002). The PKA signaling pathway activates CREM (cAMP-response element modulator) and CREB (cAMP-response element binding protein) that interact with specific regulatory regions on specific genes. The TSH receptor also activates protein kinase C (PKC) and diacylglycerol (DAG) (Spaulding 2000). Thus, activation of the TSH receptor produces a transient increase in intracellular free calcium involving an IP_3-dependent mechanism.

40. Likewise in other vertebrates, thyroid hormone is under the combined regulation of iodine availability and TSH. However, there is no evidence that toxicants can directly interfere with TSH synthesis or secretion, or directly with the ability of TSH to induce a signalling cascade in thyroid cells. However, there are indications that some toxicants can alter the TRH-induced increase in serum TSH (Khan and Hansen 2003), suggesting that this may be a point of disruption by some classes of chemicals.

3.5.2.2 Thyroglobulin Synthesis

41. Thyroglobulin (Tg) is the substrate upon which thyroid hormones are synthesized (Dunn and Dunn 2000). In its normal form, Tg is a dimer with a molecular weight of 660,000 daltons. Tg synthesis is controlled by three transcription factors – TTF-1 (thyroid transcription factor-1), TTF-2, and Pax8 (Damante and Di Lauro 1994; Kambe et al. 1996; Kambe and Seo 1996). Hypophysectomy or thyroid hormone treatment, both of which will decrease circulating levels of TSH, can decrease transcription of Tg in rats (Van Heuverswyn et al. 1984). This is believed to be a cAMP-mediated event (Dunn and Dunn 2000). The polypeptide chain of Tg is synthesized ribosomally and bound to the rough endoplasmic reticulum (Vassart 1972). Under normal circumstances, properly folded Tg dimers migrate to the Golgi complex to complete the addition of carbohydrate and sulfate moieties (Ring et al. 1987; Spiro and Spiro 1988).

42. Like TSH, Tg is conserved among vertebrates (Ogasawara et al. 1999). However, there is no evidence indicating that environmental toxicants can directly influence the production of Tg.

3.5.2.3 Regulation of Iodine Uptake

43. The thyroid gland can concentrate iodine 20-40 fold over blood levels under normal physiological conditions (Carrasco 2000). The sodium-iodide symporter (NIS) mediates the initial step in thyroid hormone synthesis – the uptake of iodide into the cell. NIS accomplishes this because it is an intrinsic plasma membrane protein on thyroid follicular cells and it couples the inward "downhill" translocation of Na+ to the inward "uphill" translocation of I-. The driving force for the process is the inwardly directed Na+/K+ ATPase that generates a large concentration gradient in sodium (35-fold higher outside the cell). NIS is blocked by the anions thiocyanate and perchlorate. Interestingly, perchlorate does not appear to be transported by the NIS (Eskandari et al. 1997; Yoshida et al. 1997; Yoshida et al. 1998), indicating that it is a blocker of NIS function, not a competitive inhibitor. A number of environmentally relevant anions also inhibit NIS function (e.g., NO_3^-, ClO_3^-, and others) (Wolff 1998). Transcription of the NIS gene is under the regulation of TTF-1, TTF-2 and Pax8; these are activated by PKA activity

stimulated by TSH. Thus, the ability of the thyroid gland to trap iodide is enhanced by TSH (up to 200 fold).

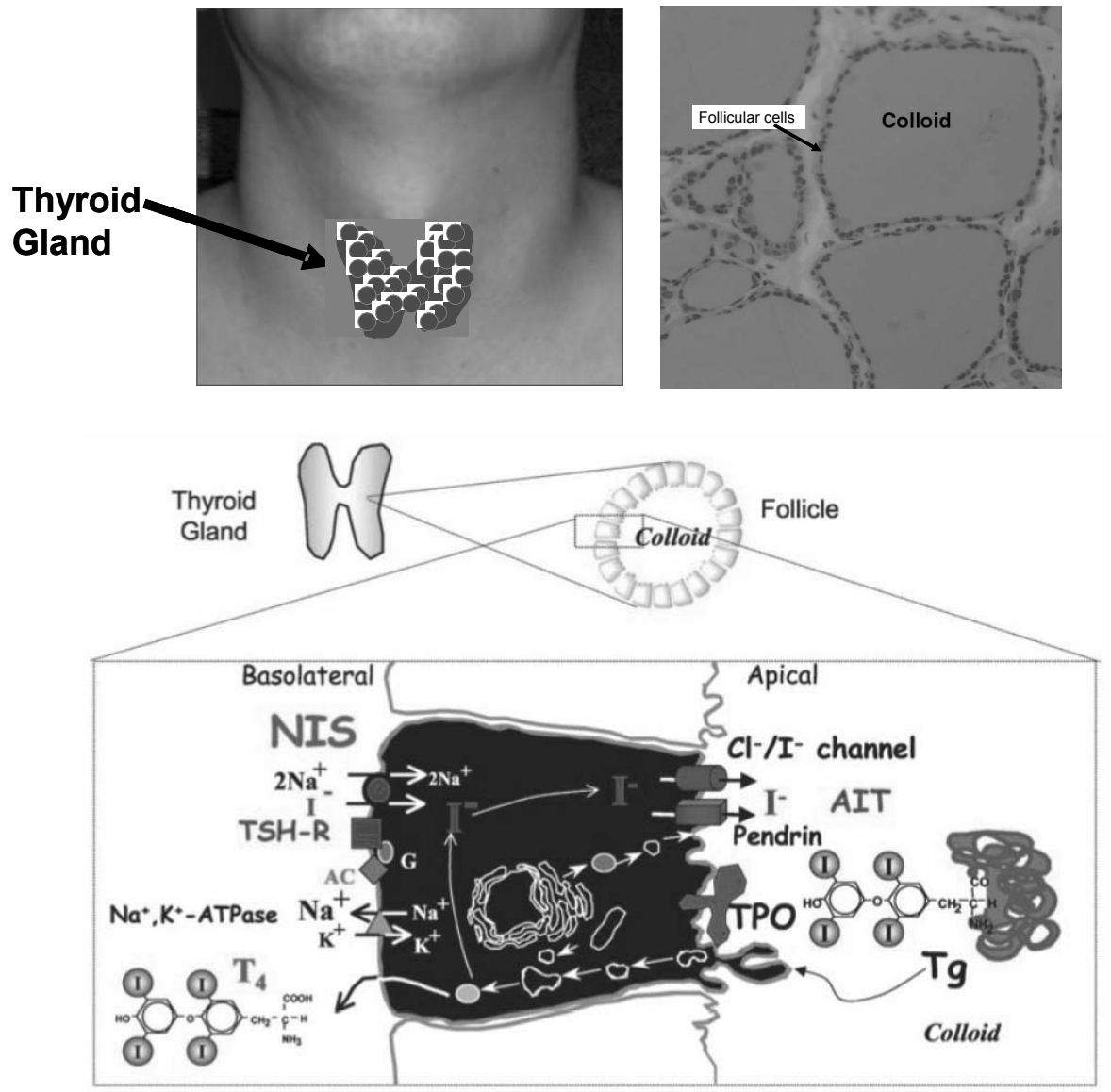
44. The NIS is also structurally and functionally conserved among vertebrates (Cabello et al. 2003). In addition, there are a number of toxicants that affect iodide uptake through this protein (Wolff 1998). Thus, this is likely to represent an important mode of action of thyroid toxicants. There is also a poorly understood relationship between mild iodine insufficiency and autoimmune thyroid disease (Laurberg et al. 2000). It is not clear whether this relationship occurs in non-human mammals, or non-mammalian vertebrates. Moreover, it is not clear whether environmental inhibitors of NIS (e.g., perchlorate) may also be related to autoimmune thyroid disease.

45. NIS expression is affected by a number of environmental factors such as iodide excess. Excessive iodine intake suppresses thyroid hormone synthesis, an effect known as the Wolf-Chaikoff effect (Wolff et al. 1949). "Escape" from the Wolf-Chaikoff effect occurs after two days, and it is likely that down-regulation of NIS expression accounts for this escape (Eng 1999). In addition, other kinds of toxicants may influence NIS expression, either directly or indirectly (Wenzel 2005).

3.5.2.4 Mechanism of Iodine Organification - Thyroperoxidase

46. There are four major sites on the thyroglobulin protein where iodine becomes covalently attached ("hormonogenic" sites designated A-D) (Dunn and Dunn 2000). These "sites" are tyrosyl residues that accept an iodine atom as the consequence of thyroperoxidase activity. The utilization of the major and minor (i.e., less frequently used) sites varies under different physiological conditions and among different species. Some of these sites are more important for the formation of T_4 compared to T_3.

Figure 3-2 Thyroid Structure and Function



The upper left panel shows the position of the thyroid gland in humans. This position is similar in all mammals, but in some fish (but not all), and in some amphibians, the thyroid follicles are distributed diffusely in the pharyngeal regions. In other animals, the gland is medial, not paired. The upper right panel shows a histological section through the human thyroid gland. Note several large follicles and interfollicular connective tissue stroma. This follicular organization is similar among all vertebrates. In the lower panel is a diagram of the follicular cell, emphasizing the role of TSH in regulating iodide uptake through the sodium/iodide symporter (NIS), pendrin and the thyroperoxidase.

47. Iodide, the form of iodine that enters the cell, must be oxidized to a higher oxidation state before it is transferred to Tg (Taurog 2000). Of the known biological oxidizing agents, only H_2O_2 and O_2 are capable of oxidizing iodide (Taurog 1964). Organification of iodine is controlled by the enzyme thyroperoxidase (TPO). TPO is a heme-containing enzyme and there are two substrate sites on the molecule. Both substrates are assumed to undergo one-electron oxidation, yielding the corresponding radical (I. and Tyr.). Thus, this is a two-step mechanism of iodination, similar to other peroxidases (e.g., lactoperoxidase). TPO has no catalytic activity in the absence of hydrogen peroxide. It is likely that the dual oxidase (DUOX) system produces this important oxidizer. TPO is also involved in the coupling reaction—the process whereby iodinated tyrosyl residues are coupled together with an ether bond (-O-) (Taurog and Nakashima 1978). The proposed coupling scheme is as follows (Taurog 2000): 1) TPO + H_2O_2 oxidize targeted tyrosyl residues on Tg, forming an oxygen radical on one residue and a carbon radical on the other, 2) there is a nonenzymatic coupling forming a 3) quinolone ether followed by a 4) rearrangement which removes the amino terminus and forming the iodothyronine.

48. The TPO enzyme is highly conserved among vertebrates. Moreover, there are a number of toxicants that directly interfere with TPO activity (Wolff 1998). The relationship between TPO inhibition and Tg iodination is not well understood (Doerge and Chang 2002; Doerge and Sheehan 2002b). Therefore, TPO inhibition itself is less likely to be a reliable endpoint for adverse effects.

3.5.2.5 Generation of Hydrogen Peroxide by the Dual Oxidase Enzymes (DUOX/ThOX)

49. A crucial step in thyroid hormone synthesis is the oxidative coupling of iodide to thyroglobulin that is catalyzed by thyroperoxidase. The limiting factor of this reaction is the generation of hydrogen peroxide that has been linked to an enzymatic system located at the apical pole of thyroid follicular cells. This enzymatic activity is thought to be exerted by NADPH oxidases encoded by two recently cloned genes h*ThOX*1 and h*ThOX*2 (Pachucki et al. 2004). Both genes are expressed at high levels in thyrocytes. Functional studies have failed to show that these dual oxidase (DUOX) enzymes function alone. In expression studies, the enzymes do not localize to the membrane and do not catalyze the generation of hydrogen peroxide. Recently, Wang et al. have identified a novel protein partner of the DUOX enzymes, which may begin to account for full activity of these enzymes (Wang et al. 2005). Proper function of these genes is physiologically important inasmuch as persistent mild hypothyroidism is associated with genetic defects in the DUOX genes (Vigone et al. 2005). Thus, it is entirely possible that xenobiotics could interfere with DUOX activity, producing a subtle adverse effect on thyroid function. However, these potential targets of thyroid toxicity have not been evaluated.

3.5.2.6 Location of Iodine Organification

50. There is autoradiographic evidence obtained by electron microscopy indicating that Tg iodination occurs at the cell-colloid interface close to the apical membrane (Ohtaki et al. 1982; Yokoyama and Taurog 1988; Taurog et al. 1990). This process occurs quickly inasmuch as at least one report demonstrates the appearance of silver grains (in liquid emulsion) concentrated over the apical border of the cell 40 seconds after injection of ^{125}I in rats (Ekholm and Wollman 1975; Wollman and Ekholm 1981). Thus, Tg appears to be iodinated on the border of the colloid and the apex of the thyroid follicle cell, and specific tyrosyl residues are coupled within the backbone structure of Tg. This is the material stored in the colloid of the thyroid follicle. These kinds of studies have not been performed in other vertebrates.

3.5.2.7 Thyroglobulin Storage

51. Iodinated Tg is stored in the adult rat thyroid gland at a high concentration (>100 mg/mL) (Smeds 1972a, 1972b) indicating that the rat stores only a few days' worth of thyroid hormone (see references in (Greer et al. 2002)) whereas the normal adult human thyroid stores perhaps several months'

worth of hormone (Brabant et al. 1992; Dunn and Dunn 2000). In contrast, the fetal and neonatal human thyroid gland contains very little iodinated Tg (Etling and Larroche 1975; Etling 1977; van den Hove et al. 1999; Savin et al. 2003), containing only enough iodinated Tg for a single day's worth of thyroid hormone at birth. This is important because chemicals that inhibit thyroid hormone synthesis (NIS- or TPO-inhibitors) would not affect thyroid hormone release until this stored material is depleted.

3.5.2.8 Thyroid Autoregulation by Iodine

52. Iodine is not only a requirement for thyroid hormone synthesis, it directly regulates many if not all the functions of the thyroid gland itself (Pisarev and Gartner 2000). The observation that serum TSH remains within a normal range despite fluctuations in daily iodine intake (in iodine-sufficient regions) of between 50 and 1,000 µg suggests that iodine is playing an autoregulatory role. Excess iodine impairs iodide organification ((Wolff et al. 1949) cited by (Pisarev and Gartner 2000)) and though this is a temporary block, children of women treated during pregnancy with the highly iodinated drug amiodarone have hypothyroidism and neurological deficiencies (Bartalena et al. 2001). In contrast, iodine deficiency leads to hypersensitivity to the goitrogenic effects of TSH (Bray 1968). In humans, mild iodine deficiency can lead to goiter in the absence of elevated levels of serum TSH (Gutekunst et al. 1986), though it must be stated that the population reference range of serum TSH is much broader than the individual variance in serum TSH (Andersen et al. 2002). Still, goiter development in geographical regions of the world with low iodine correlate better with thyroidal iodine than with serum TSH (Stubner et al. 1987).

53. Excess iodide consumption (or treatment) directly inhibits thyroid adenylate cyclase activity (Rapoport et al. 1975). This inhibitory effect of excess iodide on adenylate cyclase is itself blocked by inhibitors of iodide organification, indicating that iodinated intermediates formed by the action of thyroperoxidase play a role in regulating cAMP production (Corvilain et al. 1988). Therefore, in both humans and in experimental rodent systems, persistent exposure to excess iodide results in an inhibition of intracellular thyroidal cAMP and all cAMP-mediated events (Van Sande et al. 1975; Filetti and Rapoport 1983). The observation that excess iodide inhibits the transport of iodide, uptake of deoxyglucose and amino acids into the thyroid, as well as cAMP formation and Na/K-ATPase activity in thyroid cells indicates a membrane site of action of iodide (Krawiec et al. 1991). The mechanism(s) by which iodide controls thyroid function are not well understood. It is possible that there are iodocompounds produced by thyroperoxidase, other than thyroglobulin and thyroid hormones, which then mediate the inhibitory effects on the thyroid gland. Some have suggested that these are iodolipids, especially arachadonic acid derivatives (Krawiec et al. 1991; Dunn and Dunn 2000).

54. The direct effects of intrathyroidal iodide on thyroid function are not well understood in other vertebrates. However, these studies indicate that toxicants blocking the NIS may exert complex effects on thyroid function that are not revealed in simple measures of circulating levels of thyroid hormones.

3.5.2.9 Role of Iodine Organification and Link to Synthetic Events

55. As reviewed above, excess iodide can inhibit the activity of adenylate cyclase; therefore, iodide can block both iodine organification and synthesis. Although there are no studies that indicate a functional coupling between iodine organification (i.e., TPO activity) *per se* and hormone synthesis and release, the observation that TPO inhibitors block the autoregulatory effects of iodide is important. Within this context, several reports demonstrate that dietary iodide intake changes the vascularity of the thyroid gland. Michalkiewicz et al. 1989 was the first to show that low dietary iodine content can increase thyroidal vascularity in rats, and this is reversed by a high iodine diet. The changes occurred within 7 days (the first time examined) and remained nearly the same at 133 days. This observation was repeated for human volunteers (Arntzenius et al. 1991), showing that thyroidal blood flow (measured by Doppler analysis) is inversely related to dietary iodine intake and is independent of serum TSH. Because of the role of TPO in

the autoregulation of thyroid function, this is an issue that must be considered in experiments using TPO inhibitors.

3.5.3 Hormone Release

3.5.3.1 Regulation of Hormone Release by TSH

56. Thyroid hormones (T_4 and T_3) are stored in the colloid as part of the iodinated Tg molecule. Therefore, prior to their secretion from the thyroid gland, T_4 and T_3 must be released from the peptide linkage within Tg. In the rat, as in other species, the process of hormone release from the thyroid gland begins with activation of the TSH receptor and accumulation of cAMP. This results in endocytosis of colloid and fusion of the endosome with a lysosome. Following this, the endosomal vesicle fuses with the basolateral membrane of the thyroid follicular cell and products are released, including Tg, T_4, and T_3.

3.5.3.2 Mechanisms of Colloid Endocytosis

57. The thyroid cell responds rapidly to TSH stimulation, with pseudopodia forming on the apical surface into the colloid, followed by numerous colloid droplets inside the cell (Wetzel et al. 1965). Iodinated Tg first appears intracellularly inside apical coated vesicles (Bernier-Valentin et al. 1990). It is not clear whether this is a Tg receptor-mediated clustering of iodinated Tg, or if the pinocytotic vesicle simply ingests colloid; it may be moot because the concentration of Tg is as high as 100 mg/mL, which may be high enough that sufficient Tg is captured within a single vesicle such that further concentration is not required. Immature Tg molecules may be recognized and recycled by the thyroid cell. The evidence for this is as follows. First, Tg binds to membrane preparations made from thyroid cells; binding is pH and temperature dependent, but is not dependent on the degree of Tg iodination (Consiglio et al. 1979). In contrast, Tg binding to membrane preparations is dependent on the degree of post translational modification of Tg including addition of sialic acid and N-acetylglucosamine (Consiglio et al. 1981; Miquelis et al. 1987; Miquelis et al. 1993). These studies indicate that there is selective uptake of Tg molecules. Although Kostrouch et al. (1991, 1993) found no evidence that Tg and albumin were taken up into thyroid cells selectively, they did find that the two proteins exhibited different intracellular fates, further supporting the concept that there is a selective sorting process.

3.5.3.3 Mechanisms of Thyroxine Production and Liberation

58. Thyroid function, and the maintenance of normal levels of serum thyroid hormones, depend on cycles of synthesis of the protein prohormone thyroglobulin (Tg), which occurs in the cytoplasm along a secretory pathway, and its proteolytic degradation, which begins in the colloid and concludes following fusion of endocytotic vessicles with lysosome. Within thyroid follicles, newly synthesized Tg is transported along the secretory route to the apical plasma membrane of thyroid epithelial cells. After exocytosis, Tg is stored within the extracellular lumen of thyroid follicles in a covalently cross-linked form. Thyroid hormone liberation begins with the solubilization of Tg from this matrix of covalently cross-linked material. Soluble Tg is then subjected to limited proteolysis, leading to the rapid liberation of the thyroid hormone thyroxine (T_4). This step of utilization of Tg precedes its endocytosis and its complete degradation within lysosomes of thyroid epithelial cells.

59. *In vitro*, proteolysis of Tg is achieved by incubating with cysteine proteinases like cathepsins B and L. However, thyroid epithelial cells additionally express cathepsin K, and this protease is able to liberate T_4 from Tg by limited and extracellular proteolysis *in vitro*. These observations suggested that cathepsin K by itself, or in a combined action with the cysteine proteinases cathepsins B and L, might have an important function in the maintenance of constant levels of thyroid hormones in the blood. Friedrichs et al. have recently generated a number of cathepsin B, L, and/or K knock-out mice to study the physiological

relevance of these enzymes (Friedrichs et al. 2003). They found that the lack of expression of any single or multiple cathepsins caused a reduction in circulating levels of T_4 and altered the histological appearance of the colloid itself. Specifically, in the absence of enzymes (cathepsins B and L) that solubilize the cross-linked Tg in the colloid, the material cannot be removed from the colloid and the follicle continues to expand as the result of continued synthesis of Tg. Lysosomal enzyme transport from the trans-Golgi network (TGN) to organelles of the endocytic pathway is mediated by a post-translational process in which mannose 6-phosphate (M6P) is attached to N-linked oligosaccharides, followed by recognition by 46 kDa and 300 kDa M6P-receptors (MPRs) of the TGN pathway (Dittmer et al., 1999). Lysosomal enzymes are transported as proteolytically inactive precursors that, after uncoupling of receptor-ligand complexes, become matured by proteolytic processing within late endosomes or lysosomes. Using a Cathepsin-B—GFP fusion protein, Linke et al. (Linke et al., 2002a) find that cathepsin B is transported to endosomes/lysosomes from where it is matured to become an active peptidase. Moreover, Cathepsin B trafficking is regulated by TSH (Linke et al., 2002b) and appears to be involved in liberating T4 from Tg at the apical cell surface.

60. Clearly, the metabolic pathway required to liberate T_4 and T_3 from the Tg molecule is an important physiological event and that its potential disruption by environmental chemicals could be an important mechanism by which adverse effects of specific toxicants could occur. However, little is known about the potential vulnerability of this site of action in thyroid toxicity.

61. After endocytosis, the Tg backbone is broken down by the action of lysosomal enzymes after fusion of the endosome with a secondary lysosome (Dunn and Dunn 2000). The enzymes responsible for Tg degradation are not unique to the thyroid gland but are common lysosomal enzymes. The aspartic endopeptidase cathepsin D is a likely candidate (Dunn and Dunn 1982b, 1982a). Cysteine endopeptidases, cathepsins B, H, L, and S are also likely to be involved in the liberation of T_4 and T_3 from the Tg backbone (Nakagawa and Ohtaki 1984, 1985; Petanceska and Devi 1992; Dunn et al. 1996). There remain a number of questions regarding the relative importance of these different proteases in the liberation of T_3 and T_4 from Tg; it is also possible that different enzymes target specific hormonogenic sites on Tg (Dunn and Dunn 2000). Moreover, there is evidence that some enzymes are in the colloid. T_4 and T_3 liberated from Tg are released from the cell; iodotyrosyl residues are deiodinated by the thyroid-specific monodeiodinase (Rosenberg and Goswami 1979). Finally, some Tg is diverted into the bloodstream by transcytosis (Herzog 1983).

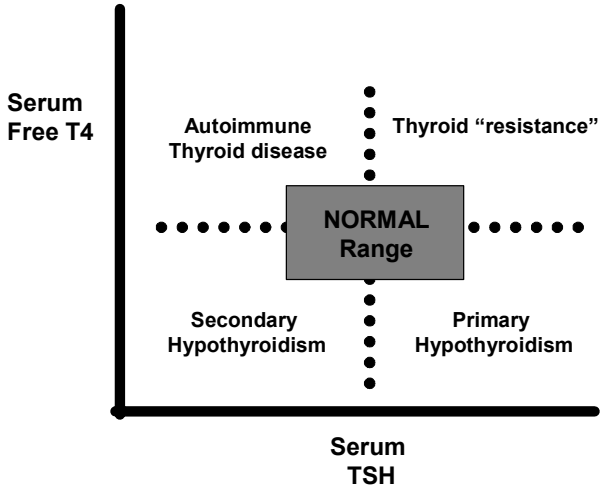
62. Although the details of the mechanics of thyroid hormone synthesis and release are likely to be similar to or identical to those described for mammals, few studies have empirically confirmed this. Likewise, there is no evidence that environmental chemicals can directly alter the steps by which iodinated Tg is stored in the colloid or taken up into the cell for processing as part of the release mechanism.

3.5.4 Regulation of Serum Thyroid Hormone

3.5.4.1 Measures of Thyroid Hormone and their Interpretation

63. Clinical measures of thyroid function have guided the measurement and interpretation of thyroid function in rodents and other experimental systems (Stockigt 2000). It is important to recognize that clinical features of thyroid disease in humans (adults, children, and newborns) are not unambiguously defined and that the biochemical markers of thyroid disease are considered diagnostic of thyroid disease itself (Stockigt 2000). Likewise in experimental systems (i.e., rodents), the overt "clinical features" of thyroid dysfunction such as body weight (or weight gain) and brain size should not be considered diagnostic of thyroid disorders even in development. Therefore, biochemical measures – serum hormone levels – should be accurately taken and reasonably interpreted. A cogent analysis of the evaluation of serum hormone levels in a clinical setting is provided by Stockigt (2000); Figure 3-3.

Figure 3-3 Relationship between Serum TSH and Serum Free T_4



See Text for Details

64. In this figure, the center rectangle defines the concentration of TSH and free T_4 that are within the normal range. Because of the negative feedback regulation of TSH by T_4, the lower right quadrant represents primary hypothyroidism (thyroid dysfunction) where T_4 is low and TSH is reflexively high. In contrast, the lower left quadrant represents secondary hypothyroidism (hypothalamic or pituitary dysfunction) in which both serum free T_4 and TSH are low. The upper left quadrant represents a TSH-independent stimulation of the thyroid gland; in humans this is most commonly associated with autoimmune Grave's disease (Schilling 1997). Finally, the upper right quadrant represents thyroid resistance in which both serum free T_4 and TSH are elevated. Although these relationships hold true in all individuals, the set-point around which TH levels are controlled appears to be narrower in individuals than in the population. Andersen et al. (2002, 2003) have found that the variation in serum T_4 and TSH in individuals is narrower than the population variance. In addition, Hansen et al. (2004) have found that genetics play a dominant role in establishing the set-point around which TH levels are regulated.

65. The logic employed as described for the clinical setting is also employed for experimental animals. However, it is important to note that the relationship shown above is for serum free T_4 rather than total T_4. The difficulty here is that the measurement of free T_4 is not simple. There are kits that measure "free T_4 index," but these measurements are known to be problematic (Midgley 2001) both because they require the assumption that the antibody used for the radioimmunoassay (RIA) has a lower affinity for thyroid hormones than do the serum binding proteins, but also because they are quite sensitive to changes in the concentration of binding proteins which are not measured in an experimental setting. This is further complicated in rodents because clinical RIAs are not always valid for rodent serum. Thus, for experimental studies, commercial RIA kits should be validated for the animal model under study (e.g., rats or mice), and the assay should be calibrated so that serum hormone levels in the subject animals are on the most sensitive part of the standard curve.

3.5.4.2 Total T_4 and T_3

66. Total T_4 and T_3 are often measured in rodents and interpreted to reflect changes in thyroid function. Most often, total T_4 is measured using a human serum-based kit (e.g., ICN Diagnostic Products). Total T_4 and total T_3 are perhaps the most variable measures of thyroid function because they vary in

relation to the amount of serum binding proteins, and in the human population, this is quite variable (Stockigt 2000). However, it is not clear whether serum binding capacity is highly variable among inbred rodent strains and, therefore, whether total hormone (T_4 or T_3) is a precise measure of changes in thyroid function. In contrast, it is often claimed that, because rodents do not have all the serum binding proteins present in humans, rodents exhibit changes in circulating levels of thyroid hormones in response to drug or chemical exposure (e.g., (Clewell et al. 2003). Although it is true that the carrying capacity of the blood increases with increasing serum binding proteins in humans [e.g., during pregnancy (Brent 1999)], there is no evidence that the lack of specific serum thyroid hormone binding proteins in rodents (i.e., thyroxine binding globulin, TBG) necessarily makes rodents more sensitive to thyroid perturbation than are humans, because this implies that rodents respond to a lower dose (per unit body weight) of a thyroid toxicant than do humans. There is little evidence that rodents are uniformly more sensitive (in general terms) to thyroid toxicants. This will likely be related to the mode of action of the toxicant. Moreover, this concept requires that we fully understand the physiological role of serum binding proteins for thyroid hormone, and this may not be the case. For example, there is evidence that the role of serum thyroxine binding proteins is to allow the equal distribution of hormone delivery to a tissue. Mendel et al. (1987) found that ^{125}I-T_4 was evenly distributed in the rodent liver following a single pass through the tissue only if serum binding proteins were present in the perfusate. However, the identity of the serum binding protein (e.g., transthyretin versus TBG) did not alter the pattern or intensity of T_4 uptake. Therefore, within the limits of sensitivity of the assay being employed (see below), total T_4 in rodents is a valid measure of thyroid function if serum binding proteins are not being affected by the treatment under study. Of course, the same precaution applies to the measurement of total T_3. Because 80% of serum T_3 is derived from peripheral deiodination of T_4, serum total T_3 is more a measure of D1 activity than it is of thyroid function.

3.5.4.3 Free T_4 and T_3

67. Serum free hormone measurements (T_4 or T_3 that is not bound to serum proteins) theoretically provide a more reliable measure of thyroid dysfunction than measures of total hormone because the latter can be altered not only by thyroid dysfunction, but also by changes in the abundance of binding proteins, which may not represent a pathological state. Normally, only approximately 0.1% of total T_4 is in the "free" form. (Stockigt 2000). However, the only direct measures of free T_3 and T_4 are the equilibrium dialysis method and the ultrafiltration method (Midgley 2001). These methods allow the direct determination of T_4 and T_3 that is not bound to serum proteins, but because the concentration of free T_4 and free T_3 is exceedingly low, large volumes of serum (e.g., 200 µl) must be used. This volume of serum can be achieved for adults, but requires pooling serum from rat/mouse fetuses or pups. Thus, several investigators have attempted to micronize this procedure using a combination of a RIA for total hormone and an estimate of the amount of free ^{125}I-T_4 (or T_3) using dialysis. This method is likely to be productive in experimental research. Moreover, in principle, if the serum binding proteins are not different among experimental treatments (which would include age since rodent TBG is developmentally regulated), then it is likely that RIA-based kits for free hormones are sufficiently reliable. Thus, measures of free T_4 and free T_3 will remain an important challenge until miconized assays using equilibrium dialysis become widely available.

3.5.4.4 Validity of Hormone RIAs for Various Compartments

68. There are two ways to check the accuracy of RIAs for specific hormones (Chard 1981). The first is to determine that a dilution series of the sample (e.g., serum) produces a curve that is parallel to the standard dilution curve. The second is that samples spiked with known amounts of hormone produce predictable increments in measured hormone. The standard human serum-based standard curve used in kits for total T_4 are not technically valid for rat serum (Gauger et al. 2004b), although the difference between the standard curve and the serum dilution curve is not large. In addition, samples must lie between two standards on the standard curve to be appropriately measured. It may be of some concern that

recently published reports using RIAs for T_4 in rats often report measures below the lowest standard (1 or 2 µg/dL and do not describe the methods used to determine the T_4 concentration, leaving the reader to assume that these values are calculated by extrapolating between the lowest standard and zero.

69. The RIA for T_3 and T_4 in tissue requires several special considerations. For example, hormones must be extracted from tissue and the extraction buffer should be matched to the tissue. Methanol is often used to extract thyroid hormones from liver, but a combination of methanol and chloroform is used to extract hormones from brain tissue. Ethanol has also been used to extract thyroid hormones from muscle and liver. In these protocols, it is important to adjust for extraction efficiency on a sample-by-sample basis (Pinna et al. 1999; 2002). Measurement of the extracted materials can be accomplished by RIA or by physical techniques such as HPLC (Morreale de Escobar et al. 1990; Porterfield and Hendrich 1992; Contempre et al. 1993).

3.5.4.5 Thyroxine Binding Proteins

70. Thyroid hormones are carried in the blood by specific proteins. In humans, about 75% of T_4 is bound to thyroxine-binding globulin (TBG), 15% is bound to transthyretin (TTR, also called "Thyroxine Binding Prealbumin" or TBPA), and the remainder is bound to albumin (Schussler 2000). TBG, the least abundant but most avid T_4 binder, is a member of a class of proteins that includes Cortisol Binding Protein (CBP) and is cleaved by serine proteases in serum (Fink et al. 1986; Khan et al. 2002). These enzymes are secreted into blood during inflammatory responses and, in the case of CBP, can induce the release of cortisol at the site of inflammation. The physiological significance of this observation is presently unclear for TBG, but it raises the possibility that TBG may selectively release T_4 under specific circumstances. Mammals differ in the specific composition of the serum proteins, which carry T_4 and, to a lesser extent, T_3. It is often stated that rodents do not have TBG (e.g., (Clewell et al. 2003), though this is incorrect (see below). Also, as described in the following chapters, all vertebrates have serum binding proteins for thyroxine, but these differ in their proportions and in their molecular structure.

3.5.4.6 Thyroid Binding Globulin (TBG)

71. In humans, TBG circulates at a concentration of approximately 0.27 mM compared to 4.6 mM for TTR and 640 mM for albumin (Robbins 2000). However, TBG carries most of the serum T_4 and T_3 because of its higher affinity for thyroid hormones (Schussler 2000). The K*i* of T_4 for TBG is $1x10^{-10}$ M and it is approximately 18.4% saturated with T_4 under euthyroid conditions; in contrast, the K*i* for TTR is 7×10^{-7} M and is approximately 0.16% saturated under euthyroid conditions. The adult nonpregnant rodent (mouse and rat) does not express TBG to a measurable extent. However, between 16 day fetus and 60 days postnatal, this pattern of TBG expression changes considerably. TBG is 2-3 times higher in fetuses than in mothers, then further increases after birth, reaching maximum values between 3 and 5 days postnatal, which are 7-8 times higher than the adult. This pattern is not correlated with the ontogenesis of TTR (Vranckx et al. 1990). In a follow-up study in rats, this group found that the mRNA encoding rat TBG in liver (cloned by Tani et al. 1994) exhibits a similar developmental pattern. In adults, TBG expression is induced by thyroidectomy in the 8 week-old male rat and T_3 replacement suppressed it. Thus, studies of toxicants that alter serum thyroid hormones may also alter TBG levels, which would be a confounding variable for their RIAs. Finally, it is paradoxical that experimental hypothyroidism causes a reduction in serum TBG levels in postnatal day (PND) 15 pups, as well as a decrease in hepatic TBG mRNA. The functional significance of these results is unclear.

3.5.4.7 Control of TBG Levels in Serum

72. In humans, serum TBG is elevated during pregnancy (Brent 1999). This is the result of the effect of the pregnancy-related increase in estrogen on the post-translational modification of TBG in the liver,

producing increased sialylation and a longer serum half-life (Ain et al. 1987). In contrast, estrogen (estradiol) does not increase TBG expression *in vitro* (Ain et al. 1988), indicating that the increased serum half-life is the most important mechanism by which TBG levels rise during pregnancy. In contrast in the rodent, estrogen does not affect TBG levels in serum (Emerson et al. 1990). Fewer studies have been focused on this issue in rodents. In rats, serum TBG levels are high during late fetal life and early postnatal development, but serum TBG levels decline to nearly undetectable levels by four weeks of age (Savu et al. 1989). Hypothyroxinemia induced experimentally in older animals (8 weeks) causes an increase in serum TBG levels as well as an increase in expression of the TBG gene in the liver (Savu et al. 1989). Interestingly, TBG is developmentally regulated in rats, with high levels circulating during early post-natal development. Moreover, there is a great deal of structural and functional microheterogeneity of rat TBG during early postnatal development. Specifically, using ^{125}I-T_4 and isoelectric focusing, Rouaze-Romet *et al.* (Rouaze-Romet et al. 1992) identified 6 labeled isoforms with isoelectric points between 4.25 and 4.55. During ontogeny, T_4 was bound to the most basic isoforms early in development and then shifted to binding more acidic forms later. The physiological significance of this is currently unknown, but it does suggest that, if TBG is a target of toxicant actions, the specific isoform attacked by the toxicant may be important.

3.5.4.8 Role of TBG in Hormone Homeostasis

73. It is clear in humans that no single serum thyroxine binding protein is essential for good health or for the maintenance of a euthyroid state (Robbins 2000). There are a number of clinical situations in which serum binding proteins are elevated or reduced (even completely absent) and the thyroid state is normal. Therefore, despite large increases or decreases in serum total T_4 and T_3 concentrations in some of these patients, serum free hormone and TSH is normal (Refetoff 1989). In contrast, there is evidence that the role of serum binding proteins such as TBG is to allow the equal distribution of hormone delivery to a tissue. Mendel (1987) found that ^{125}I-T_4 was evenly distributed in the rodent liver following a single pass through the tissue only if serum binding proteins were present in the perfusate. However, the identity of the serum binding protein (e.g., transthyretin versus TBG) did not alter the pattern or intensity of T_4 uptake.

3.5.4.9 Transthyretin (TTR)

74. Transthyretin, or thyroxine-binding prealbumin (TBPA), is, like TBG, produced in the liver and has a higher affinity for T_4 compared to T_3. In addition, TTR binds to retinol (Monaco 2000). Interestingly, TTR is also a protein involved in production of amyloid deposits (Hamilton and Benson 2001).

3.5.4.10 Control of TTR Levels in Serum

75. As reviewed above, TTR expression in liver is increased by growth hormone (GH), but not by thyroid hormone (Vranckx et al. 1994). The binding capacity of serum TTR in rats is lower in females than males, and this appears to be due to the suppressive effect of estrogen on serum TTR (Emerson et al. 1990).

3.5.4.11 Role of TTR in Hormone Homeostasis

76. TTR is present in a wide array of vertebrates, indicating indirectly that it is important in physiology (Schreiber 2002b, 2002a). Defects in the TTR gene do not produce disease in humans (Refetoff 1989; Robbins 2000). It is therefore possible that its role in physiology is more complicated than that of a simple hormone carrier.

3.5.4.12 Role of TTR in T_4 Transport to Brain

77. There is some evidence that TTR is important in transport of thyroid hormone across the blood brain barrier. In large part, this concept is derived from the observation that TTR is produced in the choroid plexus (Power et al. 2000; Zheng et al. 2001; Robbins 2002). However, this concept is not supported by the observation that mice carrying a targeted deletion of the TTR gene have normal concentrations of T_4 in the brain (Palha et al. 2000; Palha et al. 2002). Thus, if TTR plays a role in transporting T_4 across the blood-brain-barrier, it is not essential.

3.6 Thyroid Hormone Transport into Tissues

78. The preponderance of known actions of thyroid hormone are mediated by the nuclear receptors for T_3. This mechanism of action requires that thyroid hormone gain access not only to the cell, but also to the nucleus. Considering that T_4 must also be converted to T_3 prior to hormone action on nuclear receptors, it is clear that considerable coordination among circulating levels, hormone uptake into cells and conversion to T_3 must occur. The transport of T_4 and T_3 across plasma and nuclear membranes has been the subject of interest over many years. These hormones are lipophilic and were thought to diffuse passively across the plasma and nuclear membranes. However, there is good evidence for facilitated or active transport across plasma membranes, and the existence of high-affinity TH binding sites in the plasma membranes of different cells (Ekins et al. 1994; Friesema et al. 1999; Moreau et al. 1999) is important support for this concept. Oppenheimer (1983) long ago observed that ^{125}I-T_4 is taken up into different tissues at very different rates, suggesting the existence of differential transport mechanisms in different tissues. Moreover, Osty *et al.* observed the presence of a stereo-selective and saturable T_3 transport mechanism in human erythrocytes (Osty et al. 1988). There is now convincing evidence 1) of a number of stereo-selective T_4 and T_3 transporters, 2) that these transporters are selectively expressed in various tissues, and 3) that the function of these transporters are physiologically and clinically important (Friesema et al. 2005). These transporters fall into two categories. The first are the organic anionic tranport proteins (OATPs). Organic anion transport proteins represent a large family of homologous proteins, many of which have been shown to transport different iodothyronines (Hagenbuch and Meier 2004). Proteins in this family accept a wide range of ligands, not only anionic but also neutral and sometimes even cationic compounds. Some members are expressed in a single tissue, whereas others have a wider tissue distribution; moreover, some of these transporters are more selective for iodothyronines. For example, OATP1B1, 1B2 and 1B3 are expressed only in liver and have a high affinity for both T4 and T3 (Friesema et al. 2005). In contrast, OATP1C1 is expressed specifically in the brain, testis and cochlea and has a higher affinity for T_4 and reverse T_3 than for T_3 itself. In addition, OATP1C1 is expressed preferentially in endothelial cells lining the vasculature of the brain, suggesting that it is particularly important for transport across the blood-brain barrier (Friesema et al. 2005). A second class of iodothyronine transporter are members of amino acid transporters. Of particular importance are members of the monocarboxylate transporter (MCT) family. Recently, investigators have found that MCT-8 is highly selective for iodothyronines and that mutations in this gene are associated with severe neurological deficits (Friesema et al. 2004). Thus, it appears that MCT-8 is responsible for transporting T_3 into neurons and in its absence (or functional impairment), proper brain development cannot occur. The issue of thyroid hormone transport into tissues will clearly become an important focus of toxicological research. Increasingly it is becoming clear that specific compounds such as PCBs, PBDEs, Bisphenols, can bind to thyroid hormone binding proteins in serum and/or in various cellular compartments. Considering the physiological importance of MCT-8 in development, it will be important if MCT-8 function can be a target of toxicant actions. Likewise, this will be true for OATPs.

79. Recently, a nicotinamide adenine dinucleotide phosphate (NADPH)-dependent cytosolic T_3-binding protein (CTBP) has been implicated in the intracellular storage and translocation of T_3 *in vitro* (Hashizume et al. 1989; Kato et al. 1989; Nishii et al. 1989; Hashizume et al. 1991; Ichikawa and

Hashizume 1991; Kobayashi et al. 1991; Suzuki et al. 1991; Nishii et al. 1993; Mori et al. 2002; Suzuki et al. 2003b; Suzuki et al. 2003a). Furthermore, CTBP increases cytoplasmic as well as nuclear content of T_3 but paradoxically suppresses T_3-responsive gene expression in CTBP-expressing stable cell lines. These data indicate that the expression of CTBP is likely to play a fundamental role in stabilizing T_3-mediated transcription when the extracellular T_3 concentration is altered either physiologically or pathologically. Thus, it is possible that specific compounds such as PCBs could bind to these CTBPs and affect intracellular homeostasis of T_3.

80. There may be additional TH binding proteins that have not been fully explored. For example, McKinney et al. (1987) identified a T_4 binding site in rat liver nuclei. This was a relatively high-affinity binding site for T_4 but was not displaced by T_3. The identity of this binding site was not pursued.

3.7 Thyroid Hormone Metabolism

81. An important pathway by which thyroid hormones can be metabolized is deiodination. The three deiodinase enzymes catalyze this, resulting in an array of iodothyronines including T_3, T_2, reverse T_3, and T1. However, three additional pathways are important in metabolizing iodothyronines, although it is not always clear what physiological role these pathways support. The first pathway includes conjugation or glucuronidation of the phenolic hydroxy group. Conjugation includes sulfation or sulfonation catalyzed by a group of soluble sulfotransferases composed of two subunits, each with a molecular weight of approximately 34 kd. Sulfation is the transfer of a sulfonate group from the donor, 3'-phosphoadenosine 4, 5'-phosphosulfate (PAPS) and there are many different sulfotransferases that catalyze this reaction. Conjugation or glucuronidation changes the solubility of iodothyronines, allowing their concentration in bile acids and excretion through the hepatic pathway. At the same time, modification of the phenolic hydroxyl moiety changes the ability of deiodinases to act on the molecule. Thus, outer ring deiodination is blocked by sulfation or glucuronidation. An important issue is whether this pathway of TH metabolism is a target of thyroid toxicity and whether it is biologically important. Clearly, a number of TH metabolizing enzymes can be induced by various xenobiotics and this can influence circulating levels of thyroid hormone (Kretschmer and Baldwin 2005; Qatanani et al. 2005). In addition, some authors have speculated that sulfonated or glucuronidated iodothyronines can be regenerated into biologically active iodothyronines by intestinal bacteria and recycled into the circulatory system, providing a route by which serum hormone levels can be maintained. This hypothesis was not supported by the findings of Veronikis *et al.* who found that when intestinal bacterial were removed, serum TH levels were not altered (Veronikis et al. 1996). Thus, it remains to be determined that the enterohepatic pathway of TH recycling can significantly influence serum thyroid hormone levels. In addition to these pathways, oxidative deamination and ether-linked cleavage also occurs. Much less is known about the physiological role of these steps or the enzymes that carry out these reactions. As new information arises, it will be important to determine whether various toxicants can attack the thyroid system at these points of regulation. However, at this time, it is reasonable to predict that toxicant activation or inhibition of these metabolic pathways will directly influence circulating levels of thyroid hormone.

82. An additional issue that has received little attention is the possible interaction between certain classes of pharmaceuticals and environmental contaminants on circulating levels of thyroid hormone. A relevant example is that patients receiving thyroid hormone replacement therapy require an increase in their dosage if they initiate treatment with anticonvulsants (phenobarbital, phenytoin, or carbamezepine), certain antibiotics (rifampicin), or if estradiol levels increase to high levels such as during pregnancy (Zavacki and Larsen 2005). The reason the dose of T_4 replacement must be upwardly adjusted appears to be that these pharmaceuticals increase thyroid hormone metabolism in the liver. Qatanani et al. (2005) have found that, in mice, the constitutive androstane receptor (CAR) (NR1I3) is required for the increased expression of sulfo- and glucuronyl-transferases that accelerate the clearance of thyroid hormones, in turn resulting in decreased serum T_4 levels. CAR and its closely related family member, pregnane X receptor

(PXR) (NR1I2), are two of several nuclear receptor proteins known to play key roles in the metabolism and elimination of xenobiotics (Waxman 1999; Wang and LeCluyse 2003). These receptors induce cytochrome p450 (CYP) family members needed to metabolize foreign substances, such as pharmaceuticals and xenobiotics, and induce genes involved in the elimination of these compounds (Waxman 1999; Wang and LeCluyse 2003). In particular, CAR regulates the induction of many of the CYP2B family of enzymes that are highly inducible by the phenobarbital-like class of xenobiotics (Waxman 1999). Phenobarbital, and a more potent member of this group of inducers, the pesticide contaminant 1,4-bis [2-(3,5-dichloropyridyloxy)] benzene (TCPOBOP), activate murine CAR. CAR knockout mice no longer activate the cyp2B10 gene in response to phenobarbital or TCPOBOP, nor do the liver hypertrophic and hyperplastic responses elicited by these compounds occur. Qatanani *et al.* (Qatanani et al. 2005) show that CAR controls the expression of key enzymes that not only accelerate the clearance of foreign substances, but also of thyroid hormones, and that elevated serum TSH is required to maintain serum T_3 within normal limits. Thus, research to address the potential interaction between pharmaceutical treatments and environmental exposures is important.

3.7.1 The UDPGTs

83. Thyroid hormones (T_4 and T_3) are handled by the liver the way organic ions are handled – they are glucuronidated and sulfated, secreted into the biliary canaliculus, and concentrated into bile (Sellin and Vassilopoulou-Sellin 2000). The microsomal enzymes responsible for glucuronidation are the UDP-glucuronosyl transferases (UDPGTs). These phase II inducible enzymes are functionally heterogeneous. This functional heterogeneity is classically revealed in the different substrates they modify – 4-dintrophenol compared to bilirubin (Chowdhury et al. 1983). In addition, different enzyme activities are directed toward T_4 and T_3 (Hood and Klaassen 2000a), indicating the possible differential regulation of excretion of these two iodothyronines. However, there is very little information about the role of iodothyronine metabolism by liver in the regulation of serum thyroid hormone levels under normal circumstances. Moreover, there is a paucity of information about the role of these enzymes in the production of thyroid disease (hypo- or hyperthyroidism). In contrast, there is a very large literature about the role of UDPGTs in the pathway by which various microsomal enzyme inducers can cause changes in circulating levels of thyroid hormones (Barter and Klaassen 1992; Liu et al. 1995; Kolaja and Klaassen 1998; Hood et al. 1999; Hood and Klaassen 2000a, 2000b; Klaassen and Hood 2001; Zhou et al. 2001; Zhou et al. 2002; Hood et al. 2003).

3.7.2 Biliary Excretion of Thyroid Hormone

84. Oppenheimer was perhaps the first to demonstrate that drug exposure (e.g., Phenobarbital) increases the hepatic accumulation and biliary excretion of thyroid hormone (Bernstein et al. 1968). However, as stated above, there is little information about the role of these events within the normal regulation of circulating levels of thyroid hormone and it may well be revealed that this is an important area for toxicological research. It is also possible that TH metabolism can be induced by xenobiotics acting on two separate members of the orphan nuclear receptor family -- the pregnane X receptor (PXR, NR1I2; also known as SXR for steroid and xenobiotic sensor) and the constitutive androstane receptor (CAR, NR1I3). Both PXR and CAR are activated by xenobiotics and act as master regulators of phases I through III involved in the detoxification and elimination of steroids, bile acids, and xenobiotics. The purpose of phase I and II enzymes is to transform compounds into more polar forms that can be transported by phase III proteins across membranes for excretion. Detoxification genes induced by PXR and CAR include several cytochrome P450 enzymes (CYPs), enzymes such as UDPGTs, glutathione-*S*-transferases (GSTs) and sulfotransferases (SULTs), and the phase III transporters such as the multidrug resistance associated protein 2 (MRP2) and the multidrug resistance protein (MDR1). Mouse PXR is activated following exposure to pregnenolone 16 -carbonitrile (PCN, a prototypical CYP3A inducer), several pesticides, glucocorticoids and antiglucocorticoids. Human PXR is activated by xenobiotics such as rifampicin,

clotrimazole, and hyperforin, and bile acids such as lithocholic acid and 6-keto lithocholic acid. Both activation and inactivation of CAR activity can occur and inactivators are often referred to as inverse agonists since CAR has high constitutive activity *in vitro*. These pathways highlight the potential importance of an interaction between prescription drugs and environmental chemicals in affecting the thyroid system (Zavacki and Larsen 2005).

3.7.3. Deiodinases

85. There are three distinct deiodinases (Kohrle 2000). Type I (D1) and Type II (D2) deiodinases remove an iodine atom from the phenolic ring (outer ring) of the thyroid hormones. In contrast, the type III (D3) deiodinase removes an iodine atom from the amino ring (inner ring). Thus, D1 and D2 can convert T_4 to T_3, whereas D3 converts T_4 to reverse T_3. These enzymes are likely to play key roles in the control of tissue/cellular levels of T_3. Thus, different patterns of expression of these enzymes in different tissues and during development will have a major effect on the ability of thyroid hormone to affect the tissue. A good example is that of the relative contribution of D2 and D3 in tissue levels of T_3 in the human cerebral cortex and cerebellum during early development. Specifically, Kester *et al.* found that D2 levels are high in the fetal cortex, but that D3 expression is high in the cerebellum (Kester et al. 2004). In contrast, D3 levels decline and D2 levels increase in the cerebellum after birth, suggesting that these two tissues are being exposed to different levels of T_3 because of the differential expression of these enzymes during development.

86. Because the pattern of expression of deiodinases – both in different tissues and at different developmental times – may be different in different taxa or at different life stages, the effects of specific toxicants may well differ among different species. This may well be an important issue, but it has not been well studied in a toxicological context.

3.8 Thyroid Hormone Action

3.8.1 Overview of Thyroid Hormone Receptors (TRs)

87. Thyroid hormone exerts its effect on development and physiology perhaps primarily by interacting with specific nuclear proteins, the thyroid hormone receptors (TRs) (Hu and Lazar 2000; Wu et al. 2001a). Until recently, there were no putative thyroid toxicants known to bind to TRs. One early study (McKinney et al. 1987) reported that various polychlorinated biphenyls (PCBs) could bind to the "thyroxine receptor". This study was performed using rat liver nuclei, a standard procedure for measuring thyroid hormone receptor binding (Oppenheimer 1983). However, this study was performed using ^{125}I-T_4 as the ligand and they showed that this was not significantly displaced by cold T_3 (McKinney et al. 1987). Thus, although this is a potentially important observation, it is clear that they were not measuring the ability of PCBs to bind to the TR. Thus, the report by Cheek et al. (1999) was the first formal study to identify thyroid toxicants that may bind to the TR. A number of studies have now appeared, showing that chemicals such as bisphenol A (BPA, and halogenated BPA) can bind to the TR with relatively high affinity (Kitamura et al. 2002; Moriyama et al. 2002), but that parent PCBs do not (Gauger et al. 2004b). However, an individual hydroxylated PCB can bind to the TR (Cheek et al. 1999) and has been reported to cause the dissociation of liganded TR from DNA (Miyazaki et al. 2004). In addition, Yamada-Okabe et al. (2004) have shown that an individual PCB metabolite can augment, inhibit, or have no effect on T_3-mediated gene expression *in vitro* depending on the gene under study. These observations make it clear that environmental toxicants can interfere with TRs and may produce adverse effects that present as a complex mixture of effects, none of which are fully consistent with hypothyroidism or thyroid toxicity. Thus, the material below represents a background of information about the thyroid hormone receptors and the mechanisms by which they mediate hormone action.

88. TRs are members of the superfamily of ligand-dependent transcription factors (Lazar 1994; Mangelsdorf and Evans 1995; Zhang and Lazar 2000), which include receptors for steroids (estrogen, androgen, corticoids) and thyroid hormones, retinoids, and vitamin D (Lazar 1993, 1994; Mangelsdorf and Evans 1995). Two separate genes encode the TRs, designated alpha- and beta- c-*erb*A (Sap et al. 1986; Weinberger et al. 1986). Together, these two genes produce four known functional TRs: TRα1, TRβ1, TRβ2, and TRβ3 (Williams 2000; Zhang and Lazar 2000). The gene encoding TRα has 10 exons; TRα1 is composed of exons 1-9. A second major product, TRα2, is generated by the addition of a long c-terminal domain (exon 10) that disrupts the ligand-binding domain of the TR [see review by Flamant and Samarut (2003)]. Thus, TRα2 does not bind to thyroid hormone and is generally not considered to be a *bona fide* TR. Moreover, there is an internal promoter that drives the transcription of two additional short forms of the TRα gene (Chassande et al. 1997). These short forms, designated TRΔα1 and TRΔα2, are encoded by exons 8-9 and 8-10, respectively. These proteins are able to bind to thyroid hormone, but do not bind to DNA. In contrast, there are three promoters that drive the expression of the three functional TRs from the TRβ gene (Williams 2000). In addition, the TRβ3 transcript is differentially spliced to produce a TRΔβ3 isoform. Again, this small product of the TRβ gene binds to thyroid hormone, but not to DNA.

89. Triiodothyronine binds to these four *bona fide* TRs with equal, or nearly equal affinity (Oppenheimer 1983; Schwartz et al. 1992; Oppenheimer et al. 1994), although it binds with about 50 fold greater affinity than does T_4 for each receptor. Thus, T_3 is considered to be the physiologically relevant hormone on the receptor. Although T_3 binds to the various TRs with nearly equal affinity, there is good evidence that the different receptors vary enough to bind selectively to various TR analogues. For example, desethylamiodarone is a noncompetitive inhibitor of T_3 binding to the TRβ1, but a competitive inhibitor of T_3 binding to the TRα1 (Bakker et al. 1994; Beeren et al. 1995). In addition, the T_3 analog, 3,5,3'-triodothyroacetic acid (triac) has a higher affinity for TRβ1 than does T_3 but the same affinity for TRα1 as does T_3 (Schueler et al. 1990; Takeda et al. 1995; Baxter et al. 2001). A synthetic thyromimetic compound, GC-1, binds to TRβ-1 with the same affinity as T_3 but binds to TRα-1 with tenfold lower affinity (Chiellini et al. 1998; Baxter et al. 2001; Kinugawa et al. 2001). Finally, another synthetic chemical, NH-3, is a TR antagonist, but its isoform selectivity is not fully characterized (Lim et al. 2002; Nguyen et al. 2002; Webb et al. 2002). An important emerging literature focuses on the differential ability of these TH analogues to activate the various receptors on native promoters. For example, Messier and Langlois (2000) showed that triac exerts a more potent effect on TRβ1- and TRβ2-mediated transcription depending on the sequence of the TR response element (TRE). This issue is important because it is likely that xenobiotics will be found to exert these kinds of complex effects on TR activation. This issue will be expanded below.

3.8.2 Expression and Regulation of the Alpha TRs

90. It is quite likely that specific thyroid toxicants can either bind to or modulate the activity of TRs in a highly selective way (McKinney and Waller 1994, 1998). If true, the effects of these compounds will also be highly selective. Therefore, we will briefly review the literature documenting the selective expression of TRs. Thyroid hormone exerts pleiotropic effects on development and physiology. One mechanism by which thyroid hormone can exert such different, but specific, effects is for these actions to be mediated by different receptor isoforms. The differential expression of TRα and TRβ products are especially robust in the developing brain. By far, the work of Bradley et al. (1992) remain the most comprehensive mapping to date of TR isoforms in the developing rat brain. Subsequent studies have provided insight into the expression of TR isoforms discovered since Bradley's work (e.g., TRβ3). The earliest embryonic time evaluated for TRα1 expression in the fetal rat brain was E13.5 (Bradley et al. 1992). At this time, TRα1 is relatively highly expressed in the neuroepithelium that gives rise to the cortex, hippocampus, and basal telencephalon. However, it is also expressed in the trigeminal ganglion and striatum. TRα2 is expressed in these same regions and appears to be more abundant than TRα1. As

the brain expands over the next few days of embryonic development, TRα1 becomes more highly expressed in regions to which neurons are migrating and differentiating. For example, TRα1 becomes more intensely expressed in cells of the cortical plate compared to the ventricular zone. This pattern is also true for TRα2 and continues throughout fetal development. In addition, TRα1 and TRα2 are particularly abundant in the fetal thalamus, cerebellum, and inferior colliculus. At all fetal time points evaluated (E13.5, 15.5, 17.5 and 19.5), TRα2 was more abundant than TRα1, and this became more obvious in the later stages.

91. TRα isoforms are expressed in nearly all tissues of the body (Hodin et al. 1990), but have been especially studied in bone, liver, heart, and fat (in addition to brain and pituitary) (Yen 2001). The cardiovascular effects of thyroid hormone have been realized for many years. Thyroid hormone decreases vascular resistance and increases heart rate, stroke volume and contractility, with an overall increase in cardiac output (Klein and Ojamaa 1998). Thus, hyperthyroidism leads to cardiac arrhythmias and ultimately heart failure, whereas hypothyroidism leads to reduced cardiac function. This is likely to be attributed to the dominant expression of TRα1 in the heart (Hodin et al. 1990). In addition, TRα null mice exhibit decreased heart rate and contractility, but these parameters are unaffected in TRβ1 null mice (Johansson et al. 1998; Gloss et al. 2001; Weiss et al. 2002).

92. The liver is also a major site of action for thyroid hormone (McClain 1989; Song et al. 1989; Weiss et al. 1998). There are a large number of genes affected by thyroid hormone in the liver as identified by cDNA microarray (Feng et al. 2000; Yen et al. 2003). These genes may be regulated directly or indirectly by thyroid hormone. However, there are a number of genes that have been explored more extensively and are known to be regulated directly by thyroid hormone. These genes are under differential regulation by TRα and TRβ gene products. Both TRα1, TRα2, and TRβ1 are present in liver (Hodin et al. 1990; Weiss et al. 1998). Interestingly, TRα1 and TRα2 are expressed in specific cells, organized around the central vein (Zandieh-Doulabi et al. 2003). Moreover, TRα2 exhibits a daily rhythm of expression, whereas TRα1 expression is stable throughout the day. However, the physiological relevance of TRα2 expression is difficult to interpret because it does not bind to thyroid hormone.

3.8.3 Expression and Regulation of the Beta TRs

93. Bradley et al. (1992) also mapped the temporal and spatial distribution of TRβ1 and TRβ2 expression in the developing brain. The TRβ1 transcript is more widely expressed in the developing brain than the TRβ2 transcript. TRβ2 is expressed in the otic vesicle (Bradley et al. 1994), and in the upper tegmental nucleus and pituitary gland. In contrast, TRβ1 is widely expressed, though less so than TRα1 and TRα2. In early development (E13.5), TRβ1 is very low in abundance or absent from most of the brain. Cortical expression occurs by E17.5 and this is restricted to cells of the ventricular zone. During postnatal development, TRβ1 expression in the brain increases considerably (Strait et al. 1990; Bradley et al. 1992). TRβ1 expression increases first in the striatum, then in the cortex, thalamus, and olfactory bulb. TRβ2 expression is absent or nearly so in these studies. Recent studies indicate that TRα1 and TRβ1 are differentially expressed in the cerebellum (Guadano-Ferraz et al. 2003; Manzano et al. 2003b), with TRα1 expressed in cerebellar granule cells and both TRβ1 and TRα1 expressed in Purkinje cells. The regional distribution of the so-called "delta" forms of TR (TRΔα1, TRΔα2, TRΔβ3) has not been performed. TRβ1 is expressed in nearly all tissues (Hodin et al. 1990; Strait et al. 1990; Falcone et al. 1992). However, like TRα1 and TRα2, TRβ1 is regionally expressed in the liver (Zandieh Doulabi et al. 2002) and exhibits a diurnal rhythm of expression.

3.8.4 Role of Cofactors

94. The ability of TRs to affect gene transcription requires them to interact with nuclear cofactors (Glass and Rosenfeld 2000; Rosenfeld and Glass 2001; Hermanson et al. 2002; Mckenna and O'Malley 2002a). Cofactors are believed to functionally, if not physically, connect TRs with the general transcription complex (Mckenna and O'Malley 2002a), allowing hormone binding to receptors to regulate transcriptional activity. Cofactors may exert these actions by remodeling local chromatin structure. Generally, the specific recruitment of a cofactor complex with histone acetyltransferase activity may play a regulatory role in activating gene transcription, whereas the recruitment of a cofactor complex with histone deacetylase activity may play a regulatory role in gene repression (Struhl 1998).

95. A central feature of cofactors is that they can interact with a number of steroid receptors; therefore, steroid hormone receptors compete for individual cofactors indicating that cofactors can affect tissue sensitivity to the hormone. Two kinds of observations support the hypothesis that changes in cellular levels of specific cofactors can modulate cellular responsiveness to steroid/TH. First, ligand-dependent transcriptional activation by one nuclear receptor can be inhibited by ligand activation of another nuclear receptor *in vitro*, even though this second receptor does not directly regulate the affected gene (Meyer et al. 1989; Barettino et al. 1994; Zhang et al. 1996). Thus, nuclear receptors compete for specific cofactors to transduce hormonal signals to transactivate gene expression. Second, overexpression of the cofactor steroid receptor coactivator-1(SRC-1) in MCF-7 cells results in an increase in the mitogenic response to estrogen (Tai et al. 2000). Thus, the sensitivity of a cell to a specific level of hormone may be determined, in part, by the availability of specific cofactors. There are two categories of nuclear receptor cofactors in general: corepressors and coactivators (Glass and Rosenfeld 2000; Leo and Chen 2000). In the absence of TH, TRs are able to repress basal transcription via recruitment of the corepressors SMRT or NCoR (Koenig 1998; Horlein et al. 1995). In the presence of TH, TRs release their corepressor and recruit a coactivator complex that includes SRC-1 (Koenig 1998; Onate et al. 1995). The SRC family of coactivators is large and includes SRC-1, TIF2/GRIP1, and RAC3/pCIP (Leo and Chen 2000).

3.8.5 Mechanisms Controlling Pleiotropic Actions of Thyroid Hormone

96. Thyroid hormone exerts tissue-specific effects and many of these effects are also developmentally time-specific. In the case of global hypo- or hyperthyroidism, the combination of symptoms is characteristic of thyroid disease. However, thyroid toxicants that influence TR function in a selective way may present unique combinations of effects. Thus, understanding the mechanisms controlling pleiotropic actions of thyroid hormone are important for thyroid toxicology. The mechanisms by which thyroid hormone exerts such specific effects are not fully understood, but there are several lines of evidence that provide some insight. First, the spatial and temporal specificity of TR isoforms may play a major role in explaining the pleiotropic effects of TH. The α and β TRs exhibit distinct temporal and spatial patterns of expression in the developing rat CNS (discussed above) (Bradley et al. 1992). In addition, these receptors are differentially expressed in different tissues (Zandieh-Doulabi et al. 2002; 2003).

97. The differential expression of TR isoforms in different brain regions is an obvious mechanism by which thyroid hormone could regulate the expression of different genes in different brain regions. However, other factors are clearly involved in regulating thyroid hormone signaling. Observations that illustrate this point include the negative regulation of TRH expression. Thyroid hormone exerts a negative transcriptional effect on the gene encoding TRH (Hollenberg et al. 1995); however, this regulation occurs in TRH-containing neurons in the hypothalamic paraventricular nucleus (Koller et al. 1987; Zoeller et al. 1988; Zoeller et al. 1990) but not in other hypothalamic or thalamic brain regions despite the same TR being present in TRH neurons of the PVN and in TRH cells of the hypothalamus and thalamus (Koller et al. 1987; Segersen et al. 1987b). This is true also for the gene encoding RC3/Neurogranin.

RC3/Neurogranin is a well-characterized thyroid hormone-responsive gene in the developing and adult brain (Iniguez et al. 1993; Iniguez et al. 1996), and it is broadly co-expressed in the forebrain with the same TR. However, it is regulated by thyroid hormone in only a small subset of these areas (Guadano-Ferraz et al. 1997). This implies that factors other than the differential expression of TR isoform play a crucial role in regulating the temporal and spatial regulation of gene expression by thyroid hormone. It is also clear that there are mechanisms that direct specific TRs to different target genes within the same cell. For example, Monzano et al. (2003a) showed that RC3/Neurogranin is co-expressed in cells of the striatum with the gene encoding Rhes, but that their simultaneous up-regulation by TH is mediated by different receptor isoforms.

98. Finally, a major contributer to thyroid hormone action is likely to be the ability of TRs to heterodimerize with other nuclear receptors, especially the RARs and RXRs. Vitamin A metabolites such as all-*trans*-(atRA) and 9-*cis* retinoic acid (9cRA) play critical roles during embryonic development and adult physiology. At the cellular level, retinoids influence processes such as growth and differentiation by specific effects on the regulation of gene expression. Two different types of retinoid receptors have been identified. RAR is activated by both atRA and 9cRA, whereas RXR is activated only by 9cRA. RXR has been shown to regulate gene expression in response to 9cRA both as homodimers and in heterodimeric complexes with thyroid hormone receptor (TR). In these heterodimers, RXR has been shown to be allosterically blocked and function as a silent co-receptor. However, on some TREs, the RXR and TR are both responsive to their respective ligands, forming the basis for synergism between these two ligands (Botling et al. 1997). This observation indicates that xenobiotics may interfere with TR or RXR signaling and that this may selectively affect TR/RXR heterodimers at specific gene loci. This speculation requires further investigation.

3.8.6 Non-genomic Effects of Thyroid Hormone

99. Although it is generally held that most of the effects of thyroid hormone are mediated by TR regulation of target gene transcription in the nucleus, it is clear that thyroid hormones (T_4, T_3, rT_3) can exert important effects on development and physiology through non-genomic mechanisms (Davis and Davis 1996, 2002; Davis et al. 2002; Shibusawa et al. 2003b). Moreover, this may be quite important in that some toxicants may interfere with development or with thyroid hormone action in general by interfering with the non-genomic actions of TH. Early evidence for the non-genomic effects of thyroid hormone include the lack of dependence on nuclear TRs; the rapid onset of action (typically seconds to minutes); and the utilization of membrane-signaling pathways, typically involving kinases or calmodulin, that have not been implicated in direct TR function (Yen 2001). Thyroid hormone is known to influence the activity of Ca21-ATPase, adenylate cyclase, and glucose transporters (Yen 2001).

3.8.6.1 Glucose Uptake

100. Early work showed that thyroid hormone can affect glucose uptake into cells *in vitro*. For example, Segal et al. (1989) showed that T_3 causes an increased ^{14}C-2-deoxy-glucose by heart muscle *in vitro* within one minute of application. There are a number of these observations revealing a rapid effect of thyroid hormone on glucose uptake (Davis and Davis 1996). Although these studies indicate that T_3 can increase glucose uptake in the absence of new protein synthesis, it is also clear that T_3 can affect the expression of glucose transporters (e.g., GLUT 4 and GLUT 1) (Pickard et al. 1999), and targeted disruption of the TRs alters brain utilization of glucose (Itoh et al. 2001). Thus, thyroid hormone exerts both non-genomic and genomic (receptor-mediated) effects on glucose metabolism.

3.8.6.2 Effects on Mitochondria

101. Direct, non-genomic effects of thyroid hormone on mitochondria (Wrutniak-Cabello et al. 2001) may be important contributions to the physiological actions of thyroid hormone and may mediate important elements of thyroid toxicity by some classes of chemicals. There are a number of reports of rapid actions of T_3 injections into hypothyroid rodents on oxygen consumption and oxidative phosphorylation measured in mitochondria isolated from hepatocytes (Palacios-Romero and Mowbray 1979; Sterling 1986). These effects were also observed *in vitro* within 2 minutes (Sterling et al. 1977), perhaps related to the ability of thyroid hormone to stimulate the mitochondrial carrier adenine nucleotide translocase (Sterling and Brenner 1995). Thus, thyroid hormone has both long- and short-term effects on mitochondrial function.

3.8.6.3 Effects on Actin Polymerization and Vesicular Recycling

102. Leonard and Farwell (1997) demonstrated that thyroid hormone can catalyze actin polymerization in an *in vitro* system of primary astrocytes isolated from rat cerebellum. This is an important observation for several reasons. First, regulated actin polymerization is important in neurite outgrowth and cell motility (Dent and Kalil 2001). Second, Leonard and Farwell found that this activity was affected by T_4 and rT_3, but not T_3 (Leonard and Farwell 1997; Farwell and Dubord-Tomasetti 1999a). This opens an entirely novel possibility for xenobiotics that are structurally more related to T_4 than to T_3 such as polychlorinated biphenyls. Leonard and Farwell have also demonstrated that T_4 and rT_3 can stimulate vesicular transport in cells by activating myosin V motors (Stachelek et al. 2000; Stachelek et al. 2001). These effects are not mediated by the TRs (TRα1, TRβ1, TRβ2), but may be mediated by the delta isoforms (e.g., TR$\Delta\alpha$1).

3.8.7 Thyroid Hormone Negative Feedback on the Pituitary Gland

103. That thyroid hormone exerts a negative feedback effect on the pituitary is revealed principally by the negative correlation between serum thyroid hormone and serum TSH. Specifically, serum total T_4 is linearly related, with a negative slope, to the log of serum TSH (Chopra et al. 1975). This classic study by Chopra et al. focused on examining the relationship between serum TSH and serum T_4 and T_3 in patients exposed to severe iodine deficiency. Therefore, their observation that serum TSH was not at all related to serum T_3 may have been in part related to the iodine deficiency. However, many studies designed to characterize the relationship between serum TSH and serum thyroid hormones within their normal reference ranges have observed this (Spencer and Wang 1995). Thus, serum T_4 may be more important in controlling serum TSH than is T_3. This concept is supported by the observation that mice carrying a targeted deletion in the type II deiodinase (D2) causes an increase in serum T_4 and serum TSH (Schneider et al. 2001). Thus, conversion of T_4 to T_3 in the pituitary is an important step in the negative feedback action of thyroid hormone on TSH.

104. There are two important issues explored in animal studies that relate to the negative feedback of thyroid hormone on serum TSH. The first is illustrated by the work of Connors and Hedge (Connors and Hedge 1980) in which they explore the pattern of thyroid hormone replacement on serum TSH. They specifically compared tonic (i.e., continuous sc infusion delivered by an osmet minipump) versus periodic (twice daily sc injections) administration of thyroid hormone on serum TSH in the rat. They found that periodic administration of thyroid hormone was far more effective at reducing serum TSH than was the tonic administration in surgically thyroidectomized rats. However, these authors used T_3 as the replacement, and low doses of T_3 were found to increase serum TSH. Although this was paradoxical at the time, it is likely that T_3 reduced serum T_4, causing an increase in TSH. This effect of T_3 on serum T_4 may be attributable to non-genomic actions of T_3 on the pituitary (discussed below). Thus, it is difficult to draw broad conclusions about this issue, but it is likely to be of fundamental significance because the pulsatility

of hormone secretions are an important component of their actions (Brabant et al. 1990; Romijn et al. 1990; Custro et al. 1994).

105. A second issue that has been addressed both clinically and in experimental systems is the relative contribution of circulating T_4 or T_3 in the feedback regulation of TSH. The pioneering work of Chopra (Chopra et al. 1975; Chopra 1996) indicated that serum TSH is regulated by T_4, not T_3. Moreover, a number of clinical studies have appeared reporting that treatment of hypothyroid adults with a combination of T_4 and T_3 does not improve the regulation of TSH or overall outcome. In contrast, a number of reports in the experimental literature derive different conclusions. For example, Escobar-Morreale et al. (1995, 1996) demonstrated that treatment of hypothyroid rats with T_4 alone does not restore euthyroidism in all tissues. Moreover, they report that only the combined treatment of T_4 and T_3 fully restores normal levels of thyroid hormone in all tissues. Moreover, Emerson et al. (1989) reported that serum TSH in thyroidectomized rats is more highly correlated with tonic infusion of T_3 than with tonic infusion of T_4. This may represent a difference in the regulation of TSH by thyroid hormone in humans and animals, or it may represent a difference between hypothyroidism in humans and the hypothyroidism produced by goitrogens in animals, some of which affect deiodinase activity as well as hormone synthesis.

106. The mechanism by which thyroid hormone exerts a negative feedback is likely to be complex, but will also resolve the conflicting results described above. Clearly, the negative feedback effect of thyroid hormone on TSH is mediated, at least in part, by TRβ. This is shown in mice carrying targeted deletions of TRα or TRβ; the TRα null mouse exhibits normal serum T_4 and TSH, whereas the TRβ null mouse exhibits elevated T_4 and TSH (Sadow et al. 2003). In addition, people are identified with thyroid resistance syndrome on the basis of elevated T_4 in the face of normal or elevated TSH (thus, TSH is resistant to TH negative feedback. This condition is caused principally be a mutation in the TRβ receptor. Thus, the receptor-dependent negative feedback action of thyroid hormone on TSH is mediated exclusively by the TRβ isoform.

107. There are additional factors and mechanisms that influence the ability of the pituitary to release TSH and stimulate the thyroid gland. For example, TRH from the hypothalamus not only increases TSH secretion, it also appears to control glycosylation of TSH, which is important for its biological activity (Taylor and Weintraub 1985; Taylor et al. 1986; Weintraub et al. 1989; Harel et al. 1993). Therefore, when Abel et al. (1999) inserted a dominant negative mutation into the TRβ gene and expressed it specifically in the pituitary gland of transgenic mice, there was a very large increase in serum TSH, but this was not associated with a parallel increase in serum T_4 in part because TRH release was normal or reduced. Thus, the control of TSH glycosylation is an important element in the regulation of thyroid function.

108. In addition to direct effects of thyroid hormone on the gene encoding TSH (both the alpha and beta subunit) (Carr et al. 1985; Carr et al. 1987; Chin and Carr 1987; Carr and Chin 1988; Burnside et al. 1989; Carr et al. 1989), thyroid hormone also regulates the expression of receptors in pituitary thyrotropes that mediate the effects of various factors on TSH release. These include the receptors for dopamine, somatostatin, and TRH (Scanlon and Toft 2000). Finally, thyroid hormone (T_3) also appears to exert a non-genomic effect on TSH release from the pituitary gland. This appears to be related to the ability of T_3 to activate specific potassium channels in pituitary cells. This activation would hyperpolarize the cell and reduce its electrical irritability, thereby decreasing TSH secretion (Storey et al. 2002). This action appears to be mediated by direct actions on GTPases Rac and Rho.

3.8.8 Thyroid Hormone Negative Feedback on the Hypothalamus

109. Early work was unable to determine whether thyroid hormone exerted a negative feedback effect on the hypothalamus, or whether this action was directed only at the pituitary gland. There were several reasons for this. First, it was not until 1981 that Aizawa and Greer (1981) identified the specific region of

the brain that produced TRH and regulated pituitary thyrotropin secretion. The restricted distribution of hypophysiotropic TRH neurons made it difficult to test the hypothesis that TRH release was regulated by thyroid hormone in a negative feedback manner. Koller et al. (1987) and Segersen et al. (1987b) simultaneously reported on the ability of thyroid hormone to reduce cellular levels of TRH mRNA specifically in the PVN. Following these reports, a number of papers were published showing that TRH peptide is reduced in the PVN (Yamada et al. 1989) and in the pituitary-portal blood of rats (Rondeel et al. 1988). Later reports demonstrated that both T_4 and T_3 can exert a negative feedback effect on TRH mRNA in the PVN, and that this was mediated by the TRβ receptor (Dyess et al. 1988; Kakucska et al. 1992; Lechan and Kakucska 1992; Lechan et al. 1994). More recent work demonstrates that the hypothalamus is exquisitely sensitive to small changes in circulating levels of thyroid hormone (Abel et al. 1999).

3.8.9 Short-Loop and Ultra Short-loop Negative Feedback

110. The potential role of TSH in controlling TRH secretion is unclear, but is not likely to be a dominant role (Zoeller et al. 1988). In contrast, there are TSH receptors expressed in human pituitary thyrotropes (Theodoropoulou et al. 2000), indicating that TSH may play a role in its own release. Although there are TRH immunoreactive terminals contacting TRH neurons of the PVN, revealed by immunocytochemistry at the electron microscopic level (Merchenthaler and Liposits 1994), it is unclear whether TRH release exerts an ultra-short-loop feedback on hypophysiotropic TRH neurons.

3.8.10 Concept of Compensation within the HPT Axis

111. The dynamic relationship among hormones secreted by the HPT axis has the effect of maintaining serum thyroid hormone levels within a narrow range (Reichlin et al. 1972; Martin and Reichlin 1987). This observation in turn has led to the concept that the negative feedback regulation of the hypothalamic-pituitary component of the HPT axis can compensate for dysfunction of the thyroid gland in situations, for example, such as mild iodine insufficiency (Laurberg et al. 2000). Therefore, the hormone pattern of elevated TSH in the face of normal T_4 would be considered an example of compensation. This hormone pattern is defined clinically as subclinical hypothyroidism, and it is not at all clear that the interpretation for compensation is completely valid (Col et al. 2004; Surks et al. 2004). Moreover, Andersen et al. (2002) demonstrated that the range of serum T_4 in individuals is narrower than that of the general population (from which reference ranges are derived); therefore, when serum TSH is elevated and T_4 is within the population reference range, it may well be that serum T_4 is low for the individual. This observation indicates that TSH is a sensitive marker of altered thyroid hormone levels. However, it is not known whether the sensitivity of the hypothalamic-pituitary unit to thyroid hormone negative feedback is more sensitive than all other tissues to changes in serum thyroid hormone levels.

112. Therefore, the conclusion about whether changes in hormone levels represent a compensatory or adverse effect should rest on specific endpoints of thyroid hormone action that would support such a conclusion. For example, Capen clearly articulates the evidence required to determine whether the responsive increase in serum TSH following thyroid hormone insufficiency is adverse or compensatory within the context of increased risk of thyroid cancer (Capen 1994, 1997). Because the distinction between adverse and compensatory changes is related to the increased risk of thyroid cancer in response to increased proliferative capacity of the thyroid gland, the evidence required to discriminate between adverse and adaptive responses is related to measures of cell proliferation in the thyroid gland (i.e., hyperplasia versus hypertrophy). This is a very rational concept.

113. Similarly, to determine whether changes in the HPT axis are adverse or adaptive within the context of neurodevelopment requires overt measures of neurodevelopment that are specifically designed to capture effects of thyroid hormone insufficiency in the brain. Discriminating between adverse and compensatory (adaptive) changes within the HPT axis is not trivial. For example, two recent studies

demonstrate that changes in serum T_4 levels in pregnant rats can produce effects on the fetal brain without affecting TSH levels in maternal serum. Specifically, Dowling (Dowling et al. 2000; Dowling and Zoeller 2000) showed that low doses of T_4 given to hypothyroid pregnant rats (made hypothyroid with the goitrogen methimazole, MMI) can produce effects on the expression of specific genes within the developing brain without suppressing serum TSH in the maternal circulation. In addition, Auso et al. have recently reported that as little as 3 days of MMI treatment to pregnant rats can cause a transient decline in maternal T_4 of only 30%, but that there are significant migration defects in the cerebral cortex of the offspring (Auso et al. 2004a). Thus, thyroid hormone insufficiency in pregnant rats can produce effects on fetal brain development in the absence of overt measures of "compensatory" changes within the HPT axis (i.e., changes in serum TSH). This is likely to be an issue of timing in that MMI is known to cause a decrease in T_4 and an increase in TSH. However, these two events are obviously dissociable temporally.

3.8.11. Toxicant Effects on Compensatory Mechanisms in Mammals and Across Vertebrates.

114. The observation by Andersen *et al.* that the individual variance in serum TSH, T_4 and T_3 are narrower than the population variance (Andersen et al. 2002) suggests that the set-point around which thyroid hormones are regulated is slightly different among individuals. This concept has several important implications. First, it suggests that an individual may be mildly symptomatic for thyroid disease with serum T_4 and/or TSH within the population reference range. This is important from a toxicological point of view because it suggests that the definition of "adverse effects" requires measurement of endpoints of thyroid hormone action rather than solely measurements of thyroid function. Second, it suggests a great deal of complexity in the regulation of the HPT axis such that there is balance between the negative feedback system and the mechanisms delivering thyroid hormone to cells (e.g., serum proteins, deiodinases, transporters and receptors). In a Danish twin study, Hansen *et al.* (Hansen et al. 2004) found that a very large proportion (60%) of the variance in serum TSH among individuals is related to genetics. Although it is unclear exactly how genetic (heritable) traits are related to the set-point around which the HPT axis is regulated, it is possible – if not likely – that it is related to specific sequence differences in genes coding for proteins involved in various aspects of thyroid hormone synthesis, delivery and signalling. In support of this hypothesis, Peeters *et al.* recently characterized a new polymorphism in the type 2 deiodinase gene (D2) in a Danish population, finding that a polymorphism with an incidence of 30% was significantly associated with serum total and free T_4 and serum free T_3. The specific SNP is in the 5' UTR of the human D2 gene and this transition from a glycine to an asparagine caused a reduction in translation of the transcript. The subsequent reduction in serum hormone levels may be attributable to either a reduction in D2 activity in skeletal muscle, which may be a physiological source of circulating T_3, and/or it may be due to enhanced D2 activity in the pituitary gland, which is required for the negative feedback action of T_4 on pituitary TSH (serum TSH levels were not associated with this SNP).

115. These findings have important, but untested, implications for studies focused on thyroid toxicants. First, it suggests that differences – even subtle – in the structure of the various proteins involved in thyroid function, thyroid hormone delivery or thyroid hormone signalling may affect the balance of regulatory events within the HPT axis that maintains serum hormone levels within the normal range for that particular species. Thus, differences in D2 sequence either across vertebrate species or within vertebrates as a polymorphism may influence the set-point around which thyroid hormone exerts a negative feedback effect on the hypothalamus-pituitary. The issue of the set-point of negative feedback may be unique to D2 since D2 activity appears to regulate the fasting-induced reduction in hypothalamic tone to the pituitary, which mediates the reduction in serum thyroid hormone levels in this physiological state (Diano et al. 1998; Lakshmy and Rao 1999; Alkemade et al. 2005; Coppola et al. 2005). However, SNPs in all genes in the pathway of thyroid hormone synthesis, delivery and signal transduction, as well as species differences in sequences of these genes, may also underlie individual and/or species differences in the sensitivity of the HPT axis to specific toxicants. Finally, there are clearly differences among species and between vertebrate classes in the relative importance of various proteins in specific events. For

example, one receptor isoform may mediate specific events in one species that is mediated by other receptor isoforms in a different species. Thus, toxicant inhibition of a specific protein (e.g., deiodinases, receptors, etc) may have different effects in different species because the relative role of that protein in TH homeostasis may differ. Although this suggests that particular species may be employed because of these potential differences in specific vulnerabilities, we have a great deal to learn before we can make evidence-based predictions.

3.9 The Role of Thyroid Hormone in Mammalian Development

116. Thyroid hormone is essential for normal brain development in humans and in animals (Howdeshell 2002), and the consequences of exposure to thyroid hormone insufficiency during development are permanent (Zoeller and Rovet 2005). Likewise, xenobiotics may exert neurotoxic effects during development by interfering with thyroid hormone signalling. Strategically, development also offers a number of potential end-points to test chemicals for their ability to interfere with thyroid hormone signalling. Therefore, we will review the role of thyroid hormone in brain development with an emphasis on providing guidance to those charged with constructing an EDSP to capture thyroid toxicants with developmental effects.

3.9.1 Overview of Thyroid Hormone Effects in Human Brain Development

117. Studies illustrating the role of TH in brain development in humans are based primarily on investigations of children exposed to severe iodine deficiency (Boyages and Halpern 1993), of children with congenital hypothyroidism (CH) (Leneman et al. 2001; Hindmarsh 2002; Hrytsiuk et al. 2002; Rovet and Daneman 2002; Salerno et al. 2002), and by studies of cerebellar development in thyroid-deficient rodents (Koibuchi and Chin 2000b; Thompson and Potter 2000; Morte et al. 2002b; Singh et al. 2003b). More recent studies in humans (Haddow et al. 1999; Song et al. 2001; Chan and Rovet 2003; Kilby 2003) provide important new evidence showing that TH is important for early fetal brain development and that the timing and severity of TH insufficiency predict the type and severity of the cognitive deficits (Zoeller and Rovet 2005). Because these deficits presumably reflect the impact of a loss of TH on different aspects of brain development, this clinical research provides clues as to when and where TH exerts its effects in the developing brain. Concurrently, new evidence in genetic models of TH insufficiency, TH receptor deletion or mutation, and cofactor deletion (Flamant et al. 2002; Gauthier et al. 2002; Takeuchi et al. 2002; Flamant and Samarut 2003; Wondisford 2003) also provide critical insights into the potential mechanisms underlying TH action in the developing brain. Nevertheless, this research does not adequately account for many of the clinical observations in humans who lacked TH at specific times in development. While several recent reviews have comprehensively detailed many of the actions of TH in brain development in animals (Bernal 2002; Anderson et al. 2003; Bernal et al. 2003), critical knowledge is still lacking. However, information currently available is required to determine whether endpoints selected for screening putative thyroid toxicants are capable of capturing those compounds that may exert adverse effects on brain development by a thyroid hormone-related mechanism.

118. Inferences about the timing of TH action in the human brain are derived from observations on the effects of TH insufficiency during development. Although the fetal brain's supply of TH is derived from both maternal and fetal sources during the second and third trimesters of pregnancy (Morreale de Escobar et al. 1988; Morreale de Escobar et al. 1990; Calvo et al. 2002), the fetus depends entirely on TH of maternal origin during the first trimester. Conditions involving a reduced maternal TH contribution typically begin during the first trimester, whereas preterm birth, which severs the fetus from the maternal TH supply during the third trimester, produces TH insufficiency at a later developmental time. Finally, congenital hypothyroidism (CH) represents a condition with a postnatal TH insufficiency that persists until treatment is provided and takes effect. While each of these conditions is associated with impaired neurodevelopment, their different neuropsychological manifestations give clues as to the particular effects

of TH loss at different stages of early human brain development. Finally, conditions of mild thyroid disease, including hypothyroxinemia (low T_4 with normal TSH) and "subclinical hypothyroidism" (moderately high TSH with normal T_4) are also being revealed to have adverse effects on human development.

3.9.2 Maternal Hypothyroxinemia and Neurological Outcome

119. Three studies have described the consequences of low maternal TH levels during pregnancy on the cognitive functioning of the offspring. The first study, conducted in the 1960s by Man, found that the first 12 to 29 weeks of pregnancy appear to represent a critical period, when the neural substrates of abilities that depend on the visual system, as well as aspects of the motor system that also depend on vision, are particularly vulnerable to TH insufficiency (Man and Jones 1969; Man et al. 1971a; Man et al. 1971b; Man et al. 1971c; Man 1972). More recent studies by Victor Pop and colleagues found that levels of free T_4 and the presence of circulating antibodies for thyroid peroxidase (TPO) were strong predictors of mental development during infancy and IQ in childhood (Pop et al. 1995; Pop et al. 1999). These antibodies indicate two possibilities. First, their presence represents at least mild autoimmune thyroid disease in the mother. In addition, it is possible that these antibodies can interfere with fetal thyroid function, compromising the ability of the fetus to contribute its share of thyroid hormone during fetal development. Finally, the study by Haddow and his colleagues, which compared children of women with elevated levels of TSH during the second trimester to those of women with normal TSH levels, described a higher incidence of subnormal IQs (i.e., < 1SD below normal) in the offspring of hypothyroxinemic women, particularly if they were not treated during pregnancy (Haddow et al. 1999). Their children scored lower than controls on multiple aspects of cognitive functioning including auditory and visual attention, visuomotor ability, reading, and word discrimination (Haddow et al. 1999), whereas those whose mothers were treated for their hypothyroidism, albeit insufficiently because TSH levels were at 16 weeks, still had poorer visual attention and selective learning problems at school. A comparison of the results from offspring of untreated versus treated mothers, suggests that fine and graphomotor skills and reading abilities appear to be sensitive to TH insufficiency after 16 weeks gestation, whereas visual attention abilities are sensitive to TH insufficiency prior to 16 weeks (Klein and Mitchell 1999; Klein et al. 2001).

3.9.3 Clinical Hypothyroidism during Pregnancy and Neurological Outcome

120. Several case studies have described suboptimal neurological outcome in offspring of hypothyroid women. The findings have included diminished perceptual and motor skills (Pacaud et al. 1995) as well as a markedly short attention span (Francis and Riley 1987). Matsuura and Konishi (1990) reported on 23 families of treated hypothyroid women during pregnancy. Children from four of the five pregnancies involving severe hypothyroidism were developmentally delayed. Smit et al. (2000) studied a small group of infants of women with hypothyroidism diagnosed prior to pregnancy who were seemingly adequately treated. While their children indicated normal neurophysiologic and motor development, they had significantly lower mental development indices at 6 and 12 months. Others have found mild effects on specific cognitive abilities, particularly visual attention and visuospatial processing abilities, in the offspring of hypothyroid women (Rovet and Hepworth 2001b). The specific types of visual deficits appeared to reflect the timing of TH insufficiency during pregnancy (Mirabella et al. 2000).

3.9.4 Hypothyroxinemia in Preterm Infants and Neurological Outcome

121. Studies examining the consequences of hypothyroxinemia of prematurity have reported an increased incidence of cerebral palsy (Reuss et al. 1996), reduced intelligence (Reuss et al. 1994; Den Ouden et al. 1996; Lucas et al. 1996; Reuss et al. 1997; Lucas et al. 1998), and poor psychomotor abilities, particularly if the children are born extremely premature. However later-born infants, especially those born between 30 and 33 weeks gestation without neonatal risk, may also show mild neurocognitive

impairment that is associated with their reduced thyroid hormone levels. These children show associated deficits in visuospatial, fine motor (Siegel et al. 1982; Klein et al. 1989; Saigal et al. 1991; Vohr et al. 1992; Wolke and Meyer 1999), attention, memory (Sigman et al. 1986; Rose and Feldman 1987; Landry and Chapieski 1988; Hack et al. 1994; Korkman et al. 1996; Ross et al. 1996; Luciana et al. 1999), and math areas. Mirabella et al. (2000) found that one aspect of visual functioning, namely visual acuity, appears to be mildly reduced in infants born between 30 and 32 weeks gestation, and there was an inverse correlation between declining T_4 levels in their third trimester of pregnancy and later motor, visuomotor, and attention skills (Ishaik et al. 2000).

122. To determine whether hypothyroxinemia of prematurity can be corrected by exogenous administration of T_4, Van Wassenaer and colleagues randomly assigned high-risk preterm newborns to a 6-week trial of T_4 or placebo and evaluated the children at regular intervals in infancy and childhood (Briet et al. 1991). Although the treated group showed significantly higher levels of serum T_4, results of neurophysiologic (Smit et al. 1998a, 1998b) and cognitive testing showed no overall improvement (Briet et al. 1991; Van Wassenaer et al. 1997). However, stratification of the children by gestational age revealed a marked benefit of TH therapy for early neuromotor and later cognitive skills in the children born before 27 weeks (Briet et al. 1999), whereas children born at 28 or 29 weeks showed an adverse effect of such treatment. This dissociation was attributed to developmental changes between 25 and 30 weeks in the availability of deiodinase enzymes required to convert T_4 (in the medication) to T_3 (Hume et al. 1998; Briet et al. 1999). To test this hypothesis, Van Wassenaer et al. (1998) gave preterm infants past 27 weeks a single dose of T_3 12 hours after birth and found increased plasma T_3 levels for as long as 8 weeks with no clinical side effects, and this therapy was associated with improved outcome.

3.9.5 Congenital Hypothyroidism and Neurological Outcome

123. Congenital hypothyroidism (CH) represents a model of TH insufficiency occurring somewhat later in development than maternal TH insufficiencies or prematurity. CH is a disorder of newborns that affects about 1 in 3,500 newborns and was once a leading cause of mental retardation. However, since the advent of newborn screening programs, children are now being diagnosed and treated early in infancy before the appearance of associated symptomatology. As a consequence, mental retardation has been virtually eradicated (Klein 1980; Klein and Mitchell 1996). Nevertheless, affected children still experience reduced IQ levels by about 6 points on average (Derksen-Lubsen 1996) as well as mild to moderate impairments (Brooke 1995; Rovet 1999; Heyerdahl 2001) in visuospatial, motor, language, memory, and attention abilities (Fuggle et al. 1991; Rovet et al. 1992; Gottschalk et al. 1994; Kooistra et al. 1994; Kooistra et al. 1996; Rovet 1999; Connelly et al. 2001). About 20% of cases also have a mild sensorineural hearing loss (Francois et al. 1993; Rovet et al. 1996), which contributes to difficulties in initially learning to read (Rovet et al. 1996).

124. There exists among children with CH a wide degree of variability, which reflects factors associated with the disease and its management (LaFranchi 1999b). The most severe etiology is athyreosis or an absent thyroid gland, which occurs in about 25% of cases, while less severe causes include thyroid dysfunction (20 to 30% of cases), an ectopic thyroid (40% of cases), and either a central defect in hypothalamic or pituitary regulation of the thyroid or transient hypothyroidism from transplacental passage of maternal thyroid antibodies or exposure to thyroid-sensitive drugs and substances in the remaining 5 to 15% (Calaciura F 1995; Brown RS 1996). Children with athyreosis never produce any TH on their own and as a result, this condition involves a hypothyroidism that typically begins *in utero* once the maternal TH complement is no longer sufficient to meet all fetal needs (LaFranchi 1999a). These children typically have the poorest outcome and attain the lowest IQ scores (Rovet et al. 1987), more impaired nonverbal visuospatial and arithmetic abilities, as well as attentional difficulties compared to the other etiologic groups (Rovet and Hepworth 2001a).

125. Factors associated with the treatment of CH provide insight into effects of TH insufficiency that occur at a later stage of development. In general, a delay in the initiation of treatment is associated with poorer outcome (Hindmarsh 2002), particularly the development of memory, visuomotor, and language skills (Rovet et al. 1992). Since the advent of newborn screening, recommended starting dose levels have increased over the years while the issue of the optimum starting dose has yet to be resolved. Some of the abilities most affected by a low starting dose level appear to be children's memory and fine motor skills (Rovet and Ehrlich 1995). In addition, the longer it takes to achieve normalization of TH levels following the initiation of treatment, the weaker the language, fine motor, and auditory processing discrimination abilities (Rovet et al. 1992). In addition, increased selective attention and memory deficits (Song et al. 2001; Rovet and Daneman 2002) indicate that these abilities are sensitive to postnatal TH insufficiencies.

3.9.6 Epidemiology of Thyroid Dysfunction in Reproduction

126. About 0.3% of pregnancies occur to women with previously diagnosed hypothyroidism, but the majority of these women are typically under-treated because their doses of T_4 are not usually raised to match the increasing need for TH during pregnancy (Brent 1999). This increasing demand for TH during pregnancy should be met by increasing the dose of T_4 by 50% (Brent 1999). As a consequence, their infants are likely to have received an insufficient TH supply, particularly in early pregnancy before the fetal thyroid is functional. An additional 2.5% of pregnant women in North America have low levels of circulating T_4 without a concomitant increase in serum TSH (Haddow et al. 1999; Pop et al. 1999; Smit et al. 2000; Glinoer 2001; Calvo et al. 2002), a condition known as maternal hypothyroxinemia. Because these women are typically unaware of their biochemical insufficiency (attributing their mild symptoms to pregnancy) and do not receive supplemental T_4 (Mestman et al. 1995; Mestman 1999), this constitutes a large proportion of the newborn population with an inadequate TH supply during early pregnancy (Morreale de Escobar et al. 2000). Thus, studies of the offspring of women with either hypothyroidism or hypothyroxinemia during pregnancy provide critical information about the specific consequences of intrauterine TH insufficiency, particularly in early pregnancy.

3.9.7 Thyroid Function during Pregnancy

127. Thyroid function increases during pregnancy in the human (Brent 1999). This increase is manifested by an increase in serum total and free T_4. The increase in T_4 is due in part to the action of estrogen on serum thyroxine binding globulin (TBG) (Brent 1999). This effect is on the stabilization of TBG by estrogen rather than an increase in synthesis (Ain et al. 1987). In addition, chorionic gonadotropin (hCG) at high levels can stimulate the thyroid gland directly (Mestman 1998). In contrast, estrogen treatment in rats does not increase serum T_4 but rather decreases it (Emerson et al. 1990). Thus, in rats unlike humans, thyroid function is not altered to a great extent during pregnancy. It is important to recognize that there are currently no reference ranges for thyroid hormones (total or free T_4 or T_3, or TSH) in pregnancy. Thus, in studies of pregnant women, "normal" thyroid function is defined by using reference ranges established from studies of the non-pregnant population.

3.9.8 Thyroid Function during Lactation

128. There is little information about thyroid function in lactating women or in experimental animals. A recent study in humans demonstrates that the amount of thyroxine found in milk is not great enough to affect serum T_4 in nursing infants (van Wassenaer et al. 2002). Iodine is passed to the infant through the milk, although iodine levels in milk are highly variable (Dorea 2002) due to the mother's iodine consumption.

3.10 Overview of Experimental Studies on TH Action in Brain Development

3.10.1 Experimental Paradigm for Mechanistic Studies

129. Mechanistic studies of the role of TH in brain development have employed several methods for manipulating thyroid status in the dam. Some, mostly older but in some more recent, studies used ^{131}I to ablate the thyroid gland. This isotope of I is taken up into the thyroid gland and destroys the thyroid follicular cells sparing the parathyroid gland and thyroid C cells (e.g., (Fukuda et al. 1975)). Many studies have used either propylthiouracil (PTU) or methimazole (MMI) to control thyroid function. PTU has been found to directly inhibit the function of the thyroperoxidase enzyme (Engler et al. 1982), which is responsible for iodination of the tyrosine residues on thyroglobulin (Taurog 2000), a key step in thyroid hormone synthesis. In addition, PTU inhibits the type 1 5'-deiodinase (Ortega et al. 1996), which converts T_4 to T_3 in peripheral tissues. As such, PTU reduces the synthesis of nascent thyroid hormone including both T_4 and T_3, causing a dose-dependent decrease in circulating levels of thyroid hormone (St Germain and Croteau 1989). Thyroperoxidase (TPO) is a multisubstrate enzyme, which reacts first with hydrogen peroxide, forming an oxidized enzyme. This species then oxidizes iodide, the second substrate, to an enzyme-bound "active iodine", transferable to tyrosyl residues on thyroglobulin (TG) (Davidson et al. 1978). The thioureylene drugs including PTU, methimazole (MMI) and thiouracil, can inhibit TPO's ability to activate iodine and transfer it to TG (Davidson et al. 1978). However, these drugs act by different mechanisms. Specifically, PTU interacts with the "activated" iodine producing a reversible inhibition of TPO (Nagasaka and Hidaka 1976; Davidson et al. 1978), whereas MMI interacts directly with the TPO enzyme and irreversibly inhibits it. However, other investigators find that the thiourea drugs become preferentially iodinated by TPO, blocking Tg iodination in the process. This process would be a reversible inhibition of TPO action. The key event of TPO inhibition by PTU leads to a series of events within the hypothalamic-pituitary-thyroid (HPT) axis that may directly produce adverse effects or which may be surrogate markers of adverse effects. Finally, some investigators have used potassium perchlorate either alone or in combination with MMI (Lavado-Autric et al. 2003).

130. It is important to recognize that very few studies have used methods of manipulating thyroid status that produce a mild or "subclinical" thyroid hormone insufficiency. Rather, these studies largely produce severe hypothyroidism. Thus, the vast majority of research focused on identifying the role of thyroid hormone in brain development has modeled severe hypothyroidism (reviewed by (Schwartz 1983). Perhaps for this reason, the "clinical" symptoms of severe hypothyroidism in animals, including reduction in litter size, body weight, and brain size and a delay in developmental landmarks such as tooth eruption and eye-opening, have come to be viewed as cardinal developmental effects of thyroid hormone insufficiency. Therefore, by association, if these "clinical" signs are not observed, the implication is that there would be no other effects on brain development. The work by Lavado-Autric et al. (2003) (see analysis by (Zoeller 2003) is one of very few studies that used a method of manipulating maternal thyroid status that did not affect measures of litter size or weight. Thus, there are no experimental studies designed to determine what might be considered a "no-effect level" for maternal or neonatal thyroid hormone insufficiency on brain development. However, this will be an important issue to clarify as we consider the significance of maternal hypothyroxinemia or of thyroid toxicants on brain development.

3.10.2 Spontaneous Mutants That Have Informed TH Mechanisms

131. Several spontaneous mutant mouse lines have been described, which have provided basic information about the mechanisms guiding brain development and in some cases provide insight into the role of thyroid hormone in brain development. These are briefly described below.

3.10.2.1 Staggerer Mouse

132. The recessive mouse mutation staggerer (sg) disturbs the normal development of cerebellar Purkinje cells and affects certain functions of the immune system (Yoon 1972). Matysiak-Scholze and Nehls (1997) found that a mutation in the orphan nuclear receptor ROR alpha is the causative deletion in the common coding region of the ROR alpha isoforms. Of the four different isoforms of the ROR alpha gene that are generated by a combination of alternative promoter usage and exon splicing that differ in their DNA-binding properties, isoforms ROR alpha1 and ROR alpha4 are specifically coexpressed in the murine cerebellum and human cerebellum. The RORα gene is regulated by thyroid hormone and plays an important role in mediating the effect of thyroid hormone on Purkinje cell development (Matsui 1997; Koibuchi and Chin 1998; Koibuchi et al. 1999b). Studies of staggerer mice and of the ROR gene have also led to some insight into the mechanisms by which TR function is regulated by associated proteins (Vogel et al. 2000; Moraitis et al. 2002). Thus, ROR expression may be a useful marker of thyroid hormone action, disrupted by putative thyroid toxicants, that is known to be associated with adverse effects.

3.10.2.2 Reeler Mouse

133. The adult reeler phenotype is characterized not only by extreme laminar abnormalities of cell positioning in the telencephalic and cerebellar cortices, but also by relatively less extreme, though distinct, abnormal architectonics in non-cortical structures such as the inferior olive and the facial nerve nucleus (Goffinet 1984). The causative mutation is in a gene coding for reelin (Miao et al. 1994). Reelin is a large extracellular protein secreted by Cajal-Retzius neurons of the cerebral cortex that binds to membrane receptors on migrating neurons, inducing the phosphorylation of disabled homolog 1 (Dab1) and triggering an intracellular signaling cascade that appears to be important to instruct cells in their proper destination (Rice and Curran 2001). Reelin expression is reduced, and Dab1 expression is enhanced in the hypothyroid state (Alvarez-Dolado et al. 1999). Reelin is also involved in the peripheral nervous system in synapse elimination (Chih and Scheiffele 2003; Quattrocchi et al. 2003), which is necessary for controlling motor unit size in major muscle groups so that each muscle fiber receives innervations from a single motor nerve. Hypothyroid animals exhibit a longer period of polyinnervation of motor fibers during sciatic nerve reinnervation (Cuppini et al. 1996) while adult animals made hypothyroid undergo a period of motor axon sprouting and polyinnervation (Cuppini et al. 1994). Both of these observations support the possibility that thyroid hormone regulation of reelin in the peripheral nervous system is also important for synapse elimination.

3.10.2.3 Shiverer Mouse

134. The shiverer mutation is one of several spontaneous mutations in the gene encoding myelin basic protein (Mikoshiba et al. 1991; Nave 1994). Specifically, the shiverer mouse has a segment of the MBP gene missing. The hypomyelination present in shiverer (Readhead and Hood 1990; Mikoshiba et al. 1991) was similar enough to hypothyroid animals to lead to the recognition that thyroid hormone is a potent regulator of myelination (Bhat et al. 1979; Potter et al. 1984; Ibarrola and Rodriguez-Pena 1997a).

3.11 Targeted Deletions and Knock-out/in

135. Several mouse models of targeted deletions of specific genes important for thyroid function have been described. These mouse lines may prove useful in screening programs for thyroid toxicants or in developing cell lines that could be used for toxicological screens.

3.11.1 Pax8 Null Mouse

136. *Pax8* codes for a paired-box-containing protein with a highly restricted pattern of expression that is necessary for the development of the thyroid gland (Pasca di Magliano et al. 2000). The only known defect in the *Pax8*$^{-/-}$ mouse is the absence of the thyroid gland and as a result they are completely unable to synthesize thyroid hormone (Mansouri et al. 1998). These mice are healthy when given thyroid hormone and they have no defect in deiodinase activity that occurs following PTU use, no defect in parathyroid hormone or calcitonin that occurs following surgical thyroidectomy, and may not exhibit problems associated with direct effects of goitrogens such as the inhibition of neural nitric oxide synthase (Wolff and Marks 2002). For example, two papers appeared in the mid 1990s (Ueta et al. 1995b, 1995a) indicating that thyroid hormone regulates nitric oxide synthase (NOS) activity in the hypothalamus. They used PTU in these studies, which has since been shown to exert a direct action on NOS (Wolff and Marks 2002). Thus, the use of a *Pax8* deficient mouse may avoid the confounds of methods to manipulate thyroid status.

3.11.2 TR Knock-out and Knock-in Mice

137. TRβ knock-out mice (TRβ$^{-/-}$) have resistance to TH (Forrest et al. 1996a; Forrest et al. 1996b; Gauthier et al. 1999), meaning that they have elevated levels of both T_4 and TSH. In contrast, mice with deletion of the TRα1 and TRα2 isoforms (TR$^{o/o}$) are hypersensitive to TH in several of the tissues examined (McKenna and O'Malley 2002b) or less prone to the effects of TH deprivation (Morte et al. 2002a). Moreover, mice completely deficient in both TRα and TRβ (TR null) exhibit more severe resistance to TH than those lacking TRβ only (Gothe et al. 1999). Taken together, these data suggest that both isoforms play selective and overlapping roles, both centrally and peripherally, in the regulation of the HPT axis and in the control of tissue function.

138. It is important also to recognize that TR knock-out mice do not exhibit the phenotype presented by animals made hypothyroid (Wondisford 2003). Not only do TR knock-out mice not show affects of brain damage associated with hypothyroidism, but targeted deletion of specific TR isoforms can protect the brain from hypothyroidism in these strains (Morte et al. 2002a). These observations led to the hypothesis that the unliganded TR mediates the adverse consequences on brain development (and on the function of other tissues) of hypothyroidism. To test this hypothesis, Hashimoto et al. (2001) constructed a mouse carrying a TRβ gene with a targeted mutation in the ligand binding domain (TRβΔ337). This mutated TRβ is unable to bind to thyroid hormone, but remains capable of binding to DNA and to the co-repressor N-CoR. These investigators found that the TRβΔ337 mouse exhibits some of the same severe defects in brain development as observed in hypothyroid animals. The Bernal group in Madrid has begun to use these mouse lines to identify the effects of thyroid hormone on brain development that are mediated by specific TR isoforms (Guadano-Ferraz et al. 2003; Manzano et al. 2003a; Morte et al. 2003).

3.12 The Evolution of Thyroid Hormone Signaling

139. All vertebrates have a thyroid gland characterized by the development of thyroid follicles, the production of thyroid hormone, and the expression of a number of genes involved in thyroid development (differentiation) and the expression of the enzymes required to produce and trap iodide and synthesize thyroid hormone. Several recent reports exemplify the degree to which thyroid hormone signaling is evolutionarily conserved. For example, even ascidian larvae synthesize thyroxine (D'Agati and Cammarata 2005). Cells in an organ called an endostyle, a pharyngeal organ of urochordates, cephalochordates, and primitive vertebrates, concentrate iodide and produce thyroid hormones. Interestingly, this organ co-expresses the transcription factor TTF-1 and thyroperoxidase (Ogasawara 2000). In addition, injection of thyroid hormone into juvenile sand dollars accelerates their metamorphosis. Thyroid hormones (T_4 and T_3) are abundant in the planktonic algae that feeding sand dollar larvae consume (Chino et al., 1994; Heyland et al., 2004), and empirical studies show that these

hormones have profound effects on echinoderm life history traits, including developmental rate and size at settlement. Recent data also suggest that some echinoid larvae can synthesize thyroid hormones. Thyroid hormone receptors are also expressed in a number of invertebrates (Bertrand et al. 2004). Thus, a good deal of information is available upon which to conclude that thyroid hormone signalling is highly conserved throughout vertebrate evolution and is present in at least some invertebrates.

140. There are several implications of these findings that should be kept in mind. First, the molecular machinery required to produce thyroid hormone-synthesizing cells, to produce thyroid hormone itself, and to distribute the hormone to various tissues and act on nuclear receptors are likely to be highly conserved among all the vertebrates. However, there are clearly differences among these proteins too, as well as differences in the metabolic machinery that may activate or inactivate specific toxicants. Small differences, for example, in nuclear thyroid hormone receptors could make the difference between a chemical binding to it or not. The same is true for the enzymes and transporters associated with thyroid hormone action. Thus, it should not be assumed that if a chemical binds to a specific protein that it will or will not bind to another. For example, if a specific PCB congener binds to the rat TR, it doesn't necessarily mean that it will bind to the fish or human TR. Likewise, if this PCB congener does not bind to the rat TR, it does not necessarily mean that it will not bind to the fish or human TR. Comparative research studies across taxa are necessary to definitively determine how different species respond to the same chemicals, and regulatory assays that look at the effects of chemicals across taxa should be maintained until we more clearly understand the differences across vertebrate taxa.

3.13 Conclusions

141. Thyroid hormone is essential for normal development and physiology. Therefore, environmental contaminants that interfere with thyroid physiology, or with thyroid hormone action, may produce adverse consequences on normal development and physiology. As this chapter describes, the role of thyroid hormone is complex and there are many areas of the basic science of thyroid physiology and thyroid hormone action that are poorly understood. As the field evolves it will be important to periodically evaluate the regulatory assays to incorporate new endpoints or assays that may serve to identify thyroid disruption more clearly. However, there is currently enough basic information to justify a careful re-evaluation of standard protocols now employed to identify thyroid toxicants and to determine the degree to which these toxicants exert adverse health effects in animals and in humans. Moreover, there is enough information at this time to construct *in vitro* assays for thyroid disruption and to clearly articulate their strengths and weaknesses.

142. This information will be employed to review current screens and tests for thyroid toxicants in the following chapters, to evaluate their strengths and weaknesses, and to describe potential new screens and tests that may serve to inform the EDSP and the OECD's EDTA so that they can eventually regulate chemicals for the protection of human and wildlife population health. The degree to which these assays are applicable across taxa will also be evaluated.

4.0 RODENT SCREENS AND TESTS FOR THYROID TOXICANTS – CURRENT AND POTENTIAL

143. The goal of this chapter is to review current screens and tests performed in rodents for thyroid toxicants, to discuss their underlying strengths and weaknesses, and to propose additional endpoints for thyroid toxicants that are now available or are in the research and development phase, and that can overcome existing weaknesses. We first review the modes of action of known thyroid toxicants and potential *in vitro* and *in vivo* assays associated with these modes of action. We then review endpoints normally employed in screens/tests for thyroid toxicants. Finally, we review current assays – developed by the OECD, by Japanese investigators, and by the U.S. EPA, – with particular attention to ways in which these protocols can be strengthened for their ability to identify potential thyroid toxicants.

4.1 Known Mechanisms of Thyroid Toxicity

144. As discussed earlier, all chemicals classified as thyroid toxicants to date have been defined by their ability to reduce circulating levels of thyroid hormone (Brucker-Davis 1998). Thus, these chemicals alter the relationship between thyroid hormone biosynthesis and elimination such that the steady-state levels of hormones are reduced. The mode of action by which these chemicals can influence circulating levels of thyroid hormone are either focused on effects on thyroid hormone biosynthesis, or on thyroid hormone metabolism (Table 4-1). The modes of action reviewed below are germane to other taxa and are relevant to humans.

Table 4-1 Points of Disruption of Thyroid Hormone Synthesis and of Chemicals known to exert this Action

Mode of Action	Iodide Trapping	TPO Inhibition	Deiodinase Inhibition
Examples of Chemicals	Complex Anions, including C1O4, C1O3, NO3	Carbimazoles Cobalt Isoflavones including genistein	Thiouracils (e.g., PTU), PCBs, iopanoic acid
	Thiocyanate	Mercaptoimidazoles including methimazole and propylthiouracil resorcinol	Propanalol flavonoids

145. A workshop was held at Duke University in June of 1997 to bring together international experts on thyroid toxicology to review methods for screening putative thyroid toxicants (DeVito et al. 1999). The workshop focused on more than 20 assays or test systems that have been used to examine chemicals which alter synthesis, storage, transport, and catabolism of T_4 and/or T_3, assays that evaluate ligand binding and activation of the TRs, and *in vivo* assays that examine the effects of antithyroid agents and thyromimetics in mammalian and nonmammalian wildlife models. The purpose of the workshop was not to recommend a screening battery or to deal with policy issues pertaining to the use of such screens; the product of the workshop was intended to describe and evaluate the methods that are currently available or could be developed in the near future for screening and testing. To date, the paper by De Vito et al. (DeVito et al. 1999) likely remains one of the most cogent and concise descriptions of the extant assays for thyroid

toxicity (at least in rodents) and speculates on some potentially new assays. The following subsections represent modes of action by which toxicants influence thyroid endocrinology.

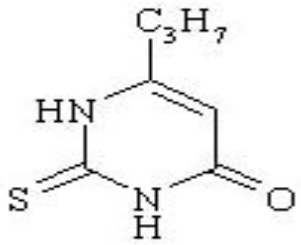
4.2 Changes in Serum Hormone Levels

146. Changes in serum concentrations of thyroid hormones (T_4, T_3, and TSH) can be caused by chemicals that inhibit thyroid hormone synthesis, release, and transport, and by chemicals that increase metabolism of various thyroid hormones (e.g., Deiodinases, UDPGTs). If a chemical decreases serum hormone concentrations, specific assays can be used to determine the mechanism by which these hormone concentrations are decreased. Moreover, the specific profile of these changes in hormone concentrations may be informative. For example, inhibition of D1 may preferentially reduce circulating levels of T_3, which may not be accompanied by a reduction in serum T_4 or TSH. In contrast, inhibition of iodine uptake is predicted to cause a reduction of T_4, leading to a decrease in both T_4 and T_3 and an increase in serum TSH. However, it is important to keep in mind that interpreting changes in hormone levels in terms of mechanisms of toxicant action or potential adverse effects is quite complex. For example, if thyroid hormones are decreased and TSH is elevated, it is important to avoid assumptions about compensatory actions. As will be described below, recent studies in rats demonstrate that goitrogens can produce effects on the fetal brain at concentrations below that which affects maternal serum TSH. Moreover, exogenous thyroid hormone can influence fetal brain measurements before it down-regulates maternal serum TSH.

4.2.1 Thyroperoxidase Inhibitors

147. 6-Propyl-2-thiouracil (PTU) is a thioamide drug that has been intensively studied in animals and in humans and is used therapeutically to treat patients with Graves' Disease (Zoeller and Crofton 2005; Cooper 2003). As a drug, it does not exist in nature and there are no environmental sources of PTU. However, as a chemical class, it represents compounds found in the environment that can affect thyroid function. PTU is well known to reduce circulating levels of T_4 and T_3 and to increase circulating levels of TSH (e.g., (Frumess and Larsen 1975; Sato et al. 1976) and has been extensively used in mechanistic research focused on identifying the role of thyroid hormone in brain development. The ability of PTU to reduce circulating thyroid hormone levels has been exploited in the treatment of hyperthyroidism in humans, including in pregnant and lactating women (Mestman 1998). PTU (Figure 4-1) is generally believed to produce deleterious effects in animals by causing a dose-dependent reduction in circulating levels of thyroid hormone. This reduction is caused by the ability of PTU to directly inhibit the function of the thyroperoxidase enzyme (Engler et al. 1982), which is responsible for iodination of the tyrosine residues on thyroglobulin (Taurog 2000), a key step in thyroid hormone synthesis. In addition, PTU inhibits the type 1 5'-deiodinase (Ortega et al. 1996), which converts T_4 to T_3 in peripheral tissues. As such, PTU reduces the synthesis of nascent thyroid hormone including both T_4 and T_3, causing a dose-dependent decrease in circulating levels of thyroid hormone (St Germain and Croteau 1989).

Figure 4-1 Propylthiouracil



148. Thyroperoxidase is a multisubstrate enzyme, which reacts first with hydrogen peroxide, forming an oxidized enzyme. This species then oxidizes iodide, the second substrate, to an enzyme-bound "active iodine," transferable to tyrosyl residues on thyroglobulin (TG) (Davidson et al. 1978). The thioureylene drugs, including PTU, methimazole (MMI) and thiouracil, can inhibit TPO's ability to activate iodine and transfer it to TG (Davidson et al. 1978). However, these drugs act by different mechanisms. Specifically, PTU interacts with the "activated" iodine producing a reversible inhibition of TPO (Nagasaka and Hidaka 1976; Davidson et al. 1978), whereas MMI interacts directly with the TPO enzyme and irreversibly inhibits it. The key event of TPO inhibition by PTU leads to a series of events within the hypothalamic-pituitary-thyroid (HPT) axis that may directly produce adverse effects or which may be surrogate markers of adverse effects. No other modes of action have been proposed for the ability of PTU to reduce circulating levels of thyroid hormone or to affect thyroid histopathology. However, a recent study indicates that PTU can exert direct actions on the activity of the neuronal isoform of nitric oxide synthase (Wolff and Marks 2002). Considering the importance of neuronal NOS in brain development and in neuronal plasticity (Blackshaw et al. 2003), it is possible that this direct action may influence brain development.

149. A good example of TPO inhibitors are the isoflavones, especially those found in soy protein (e.g., genistien, coumesterol) (reviewed by (Doerge and Sheehan 2002a). In humans, goiter has been reported in infants fed soy formula (Labib et al. 1989; Chorazy et al. 1995; Jabbar et al. 1997). In addition, teenage children diagnosed with autoimmune thyroid disease were found to have twice the rate of occurrence if they had consumed soy formula as infants (Fort et al. 1990). Boker et al. (Boker et al. 2002) recently reviewed the dietary sources of a variety of isoflavones (see Table 4-2), showing that these are common dietary components. These isoflavones are also so-called "phytoestrogens," which are highly enriched in some commercial preparations.

150. The TPO assay itself involves monitoring the iodination reaction using bovine serum albumin or tyrosine as substrates (Divi and Doerge 1996). In addition, the oxidation of guaiacol can be used as an indicator of thyroid peroxidase activity (Divi and Doerge 1994). All chemicals that inhibit the iodination reaction also inhibit the coupling reaction (Divi and Doerge 1994). The coupling reaction can be assayed using human low iodine thyroglobulin or preiodinated casein as substrates.

151. A disadvantage of the TPO assay is that purified TPO is not readily available commercially. It was previously reported that porcine TPO is the only purified preparation available (DeVito et al. 1999). Moreover, a recent on-line search of possible commercial products revealed none. However, if this assay were an important component of a chemical screening program, recombinant enzymes from different species could be developed. In fact, a strength of the TPO assay is that the sensitivity to chemical inhibition of TPO from human and experimental animals can be directly examined. *In vitro* studies have shown that TPOs from different mammals exhibit differences in their sensitivity to inhibition by propylthiouracil (PTU) and sulfamethazine (Takayama et al. 1986). Comparisons of the relative sensitivity

of TPO across species to various toxicants may assist in risk assessment for chemicals that inhibit TPO activity, although differences in the pharmacokinetics/dynamics in various species would not be captured by this *in vitro* approach. The iodination and coupling assays are specific for chemicals that inhibit TH synthesis and are unlikely to produce false positives. However, used alone as a screen, these assays have high potential for false negatives, as chemicals that alter TH concentrations through other mechanisms would not be detected. These assays have been performed for many years, are well established in the scientific literature, and numerous chemicals have been tested using these assays. Although there are no published methodologies that can be defined as high throughput screens, modification of this assay into a high throughput screen is under development in several laboratories (DeVito et al. 1999).

Table 4-2 Intakes of Phytoestrogen by Food Groups by Dutch Women

Food group	Daidzein	Genistein	Formononetin	Biochanin A	Coumesterol	Matairesinol	Secoisolariciresinol
				% daily intake			
Vegetables	31.8[1]	31.0[1]	49.8[1]	35.2	97.2[1]	6.4	8.2
Peas/beans	28.6	25.7	49.8	35.2	62.2	<0.1	0.3
Potatoes	2.1	4.1	—	—	—	4.8	5.6
Leafy vegetables[2]	0.6	0.4				1.1	1.9
Other	0.5	0.8	<0.1	—	35.0	0.5	0.4
Fruit	4.3	2.1	—	—	—	3.6	1.0
Berries	0.1	0.8				2.8	4.1
Non-berries	4.2	1.3				0.8	9.9
Fruit/vegetable juice	1.5	<0.1	—	—	—	0.3	1.6
Fruit juices	1.0	<0.1				0.2	1.5
Vegetable juices	0.5	<0.1				0.1	0.1
Coffee/tea	16.3	4.8	24.3	—	—	12.2	22.8
Coffee	14.5	4.8	24.3			—	15.8
Tea	1.8	—	—			12.2	7.0
Traditional soy foods	6.5	6.5	—	—	—	—	—
Breakfast cereals	17.2	14.4	0.1	0.1	0.2	7.0	0.1
Grain products	15.5	11.9	6.2	0.1	2.3	62.9[1]	40.8[1]
Bread	15.4	11.8	6.2	0.1	2.3	54.2	40.7
Cakes/cookies	0.1	0.1	—	—	—	5.5	<0.1
Pasta/rice	—	—	—	—	—	3.2	0.1
Nuts (mostly peanuts)	3.8	16.2	2.1	45.0[1]	—	0.1	4.8
Alcohol	<0.1	<0.1	<0.1	—	—	6.4	1.3
Other	3.1	13.1	17.5	19.6	0.3	1.1	6.4
Total	100.0	100.0	100.0	100.0	100.0	100.0	100.0

[1] Main sources (by foodgroups) for isoflavones, coumestans and lignans intake.
[2] Leafy vegetables = cabbage/lettuce/chicory/endive/spinach.

Source: Boker et al. (2002, J. Nutr. vol. 132, pp. 1319-1328). Reprinted by permission of the American Society for Nutritional Sciences.

4.2.2 Perchlorate Discharge Test

152. Perchlorate competes with iodide for thyroid uptake and also promotes the efflux of iodide from follicular cells (Atterwill et al. 1987). The perchlorate discharge test has been used for decades in both animals and humans to detect iodide organification defects (Wolff 1998; Meller and Becker 2002). In this assay, animals are exposed to a test chemical and then administered ^{125}I followed by perchlorate. Accumulation of ^{125}I in the thyroid is determined before and after administration of perchlorate. Perchlorate promotes the release of iodine that has not been incorporated into thyroglobulin. Therefore, if a chemical inhibits or deactivates thyroid peroxidase, there would be a brisk decrease in the accumulation of ^{125}I in the thyroid gland. This assay has the potential for providing mechanistic information on the actions of chemicals that alter thyroid function, but it does not necessarily meet the requirements of a screen (DeVito et al. 1999). A modification of the perchlorate discharge test that would test for chemicals that interfere with iodine uptake would be the use of thyroid scintigraphy (e.g., Schellingerhout et al. 2002). This technique is essentially that of radioactive iodine uptake inhibition used by Greer et al. (Greer et al. 2002).

4.2.3 Inhibitors of Iodide Uptake

4.2.3.1 Sodium/Iodide Symporter

153. A variety of complex anions can inhibit iodide uptake through the sodium/iodide symporter (NIS) (Wolff 1998). The defining characteristic of iodide transport is its very high specificity for iodide with respect to the chloride ion, which is abundant in biological systems. However, despite this, iodide is not the only ion selected by the NIS, nor is it the most avid (Wolff 1998). The following potency series for anions capable of blocking iodide uptake was constructed by Wolff and reviewed later (Wolff 1998): $TcO_4 \geq ClO_4 > ReO_4 > SCN > BF_4 > I > NO_3 > Br > Cl$. Although nitrate is actually less potent than iodide at the NIS, environmental contamination with nitrate has nevertheless been associated with goiter (Gatseva et al. 1998; Vladeva et al. 2000). Perchlorate (ClO_4) contamination also has been studied for its effects on thyroid function, especially considering its potency at inhibiting iodide uptake into the thyroid gland (Urbansky 2002; Strawson et al. 2004). The only epidemiological study focused on non-neonates (Crump et al. 2000) indicates that exposure to perchlorate in drinking water, in combination with elevated iodine intake, tends to increase circulating levels of thyroid hormone rather than decrease it. This observation was also observed in mice (Thuett et al. 2002). Genetic defects in NIS result in congenital iodide deficiency and congenital hypothyroidism (De La Vieja et al. 2004, 2005). Interestingly, the specific defects do not simply block the ability of NIS to take up iodide, but block its targeting to the plasma membrane, presumably by causing protein misfolding. Finally, a recent report (Breous et al., 2005) shows that environmental chemicals (phthalates) increase NIS expression by directly acting on the NIS promoter in a defined expression system.

4.2.3.2 Inhibitors of Pendrin

154. Pendrin is a protein identified by positional cloning, revealing a genetic defect resulting in Pendred Syndrome. This syndrome is one of the most common causes of profound sensorineural hearing loss and thyroid goiter (Pendred, 1896; Reardon et al. 1977; Taylor et al. 2002). Interestingly, the pendrin protein is expressed in a highly specific manner: in the thyroid gland, the kidney and in the inner ear (Everett et al. 1997; Everett and Green 1999). It is not completely clear how this expression pattern accounts for the symptoms of the syndrome. The Pendrin protein transports iodide from the apex of the thyroid follicular cells into the colloid (Figure 3-2, presented above), and it also appears to account for the iodide *efflux* from the thyroid gland upon perchlorate administration (i.e., the perchlorate discharge test) (Yoshida et al. 2002), which is why iodide efflux is exacerbated in Pendred's syndrome (Reardon et al. 1977; Reardon et al. 1999). Pendrin has a high degree of structural similarity to known sulfate transporters, but it transports iodide and chloride, not sulfate (Fugazzola et al. 2001). Although it is possible that Pendrin is a site of action for some xenobiotic chemicals, there is no information on this.

4.2.3.3 Inhibitors of TSH effectiveness

155. There is limited, but important evidence that some toxicants can influence the potency of TSH on its receptor *in vitro* (Santini et al. 2003). This may be especially important in situations of low iodine because it is clear that changes in iodine status can influence the efficacy of TSH on its receptor.

4.2.4 Xenobiotic Effects on Iodothyronine Deiodinases

156. Few studies have focused on the ability of environmental toxicants to interfere with thyroid hormone metabolism by deiodinases. However, this may be an important mechanism by which environmental chemicals could interfere with thyroid hormone action on tissues considering recent evidence that these enzymes play an important role in controlling tissue sensitivity to thyroid hormone, especially during development.

157. The development of the mammalian brain is characterized by an orderly sequence of events (Cowan et al. 1997). Moreover, the relative timing of maturational events within the brain is quite similar among mammalian species (Clancy et al. 2001). Recent work in both humans and experimental animals demonstrates that thyroid hormone exerts effects on the developing brain throughout a broad period of fetal and neonatal development (Chan and Rovet 2003), and that the developmental events and brain structures affected by thyroid hormone differ as development proceeds. Therefore, it is possible that the human brain uses a strategy for "timing" thyroid hormone sensitivity of different brain regions that is similar to that used by *Xenopus* (reviewed below). The work by Kester et al. (2004) represents a key observation suggesting that this is indeed the case.

158. Kester et al. (2004) report that in several brain regions in humans – especially the cerebral cortex – levels of T_3 increase during fetal development and this is correlated with an increase in the activity of type 2 deiodinase (D2) while the activity of the type 3 deiodinase (D3) is low to undetectable. Type 2 deiodinase controls the conversion of T_4 to the hormonally active T_3, but D3 controls the conversion of T_4 to the hormonally inactive reverse T_3. Because T_3 levels in the fetal cerebral cortex increased to an extent that could not be accounted for simply on the basis of the age-dependent increase in T_4, it indicates that D2 is causing the age-dependent increase in T_3 from 14 to 20 weeks gestation. Importantly, during this same period, the fetal cerebellum has high levels of D3 and low levels of T_3. Finally, at later gestational ages, D3 activity in the cerebellum declines and T_3 levels increase.

159. Deiodinase expression responds to changes in circulating levels of TH (Burmeister et al. 1997). Thus, thyroid toxicants may affect the ability of tissues to compensate for changes in circulating levels of thyroid hormone (Hood and Klaassen 2000b; Meerts et al. 2002). Moreover, deiodinase activities may be regulated in a complex manner that is related to both T_4 and T_3 availability in the serum (Burmeister et al. 1997). Thus, the shape of the dose-response curve defining the effect of toxicant on serum TH levels may not be parallel to the dose-response curve defining the effect of toxicant on endpoints of TH action in tissues.

160. In mammals, approximately 80% of the circulating T_3 is derived from peripheral deiodination of T_4 (St Germain and Galton 1997). As reviewed above, the deiodinases may control tissue sensitivity to thyroid hormone. For example, a recent report indicates that the human fetal cortex contains high levels of T_3 associated with high D2 activity and low D3 activity (Auso et al. 2004a). In contrast, the human cerebellum exhibited low levels of T_3 before birth, and this was associated with low D2 and high D3. Thus, it is possible that xenobiotic chemicals that alter deiodinase activity may affect thyroid hormone signaling in the developing brain or in adult tissues, thereby producing an adverse effect, but may not produce changes in serum hormone concentrations. Deiodinase assays have been used for decades to understand the metabolism of thyroid hormones and may be amenable to high throughput assays. However, because the activity of these enzymes is dependent on the serum concentrations of these hormones, these assays would be sensitive toward chemicals that alter serum TH concentrations. Moreover, alterations in deiodinase activity also may alter serum TH concentrations. If serum TH concentrations are changed by deiodinase inhibitors, it may be easier to measure serum TH concentrations than it is to determine deiodinase activity. Similar to many of the assays described above, these assays have greater utility in understanding the mechanism of action of a chemical rather than as an initial screen.

4.2.5 Toxicant Effects on Thyroid Hormone Clearance

161. Oppenheimer's group was among the first to examine the ability of chemicals (phenobarbital and chlordane) to enhance biliary secretion of thyroxine (Bernstein et al. 1968). These seminal studies were the first to show that chemicals could activate the liver to trap thyroid hormones, enhancing their elimination through the bile and decreasing their serum half-life. Research in this area has focused on the ability of chemicals to induce liver enzymes that conjugate T_4 or T_3 to glucuronide, and/or the ability of

chemicals to displace thyroid hormones from their serum binding proteins. However, there is not a consensus about the mechanism by which these chemicals, which do not act on the thyroid directly, can reduce circulating levels of thyroid hormones.

4.2.5.1 Role of Liver UDPGTs

162. Thyroid hormones (T_4 and T_3) are handled by the liver the way organic ions are handled – they are glucuronidated and sulfated, secreted into the biliary canaliculus, and concentrated into bile (Sellin and Vassilopoulou-Sellin 2000). The microsomal enzymes responsible for this activity are the UDP-glucuronosyl transferases (UDPGTs). These phase II inducible enzymes are functionally heterogeneous. This functional heterogeneity is classically revealed in the different substrates they modify – 4-dintrophenol compared to bilirubin (Chowdhury et al. 1983). In addition, different enzyme activities are directed toward T_4 and T_3 (Hood and Klaassen 2000a), indicating the possible differential regulation of excretion of these two iodothyronines. However, there is very little information about the role of iodothyronine metabolism by liver in the regulation of serum thyroid hormone levels under normal circumstances. Moreover, there is a paucity of information about the role of these enzymes in the production of thyroid disease (hypo- or hyperthyroidism). In contrast, there is a very large literature about the role of UDPGTs in the pathway by which various microsomal enzyme inducers can cause changes in circulating levels of thyroid hormones (Barter and Klaassen 1992; Liu et al. 1995; Kolaja and Klaassen 1998; Hood et al. 1999; Hood and Klaassen 2000a, 2000b; Klaassen and Hood 2001; Zhou et al. 2001; Zhou et al. 2002; Hood et al. 2003).

163. An example of the key questions regarding the role of UDPGTs in mediating toxicant effects on serum thyroid hormone levels is provided by the effect of polychlorinated biphenyls (PCBs) on serum thyroid hormone. The chlorinated biphenyl 3,3',4,4',5-pentachlorobiphenyl, Aroclor 1254, and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in rats are all known to reduce circulating T_4 (Barter and Klaassen 1992; van Birgelen et al. 1994; Schuur et al. 1998), perhaps because of their ability to induce T_4-UDPGT (Saito et al. 1991; Barter and Klaassen 1992; de Sandro et al. 1992). However, the degree to which these chemicals reduce serum T_4 is not correlated with the increase in T_4-UDP-GT activity (de Sandro et al. 1992; Hood et al. 2003). In addition, Kenechlor-500 reduces circulating levels of T_4 in both rats and mice, but induces UDP-GT in rats but not mice (Kato et al. 2003). In addition, Kenechlor-500 induces a decrease in circulating levels of T_4 in Gunn rats, a strain that is deficient in UDPGT1A isoforms (Kato et al. 2004). Thus, there is an argument that UDPGT induction alone is not a uniform marker of the ability of chemicals to cause a reduction in serum thyroid hormone. Nonetheless, the ability of chemicals to reduce circulating levels of thyroid hormone can be associated with UDPGT induction and an increase in fecal elimination of T_4 (de Sandro et al. 1992; Vansell and Klaassen 2001).

4.2.5.2 Glucuronidation Assays

164. Glucuronidation, sulfation and sulfonation followed by biliary elimination of T_4 is one of the major pathways for removing T_4 from the circulation. In both humans and rodents, there is evidence of all three mechanisms of T_4 elimination. There are at least three isoforms of UDPGT in mammals that glucuronidate T_4 (Visser et al. 1993). Several classes of chemicals induce UDPGTs responsible for the glucuronidation of T_4 (Matsumoto et al. 2002; Meerts et al. 2002; Wade et al. 2002; Zhou et al. 2002; Hood et al. 2003; Kato et al. 2003). Induction of T_4 glucuronidation increases clearance and decreases serum concentrations of T_4. Induction of T_4 glucuronidation is typically determined in hepatic microsomes from animals treated with test chemicals. These assays have been performed for decades in numerous laboratories throughout the world. These *ex vivo* assays require several days of dosing of the test chemical. The advantage of this type of assay is that it is responsive to metabolic activation of the test chemical because exposure occurs *in vivo*. The activity of hepatic microsomal T_4 glucuronidation is not as sensitive to stress and circadian rhythms as are measurements of serum TH concentrations. The disadvantage is that

these assays are not developed for use as high throughput screening tests and at present are laborious. Additionally, although these assays provide data useful in understanding the mechanisms of action, not all chemicals that affect the thyroid produce alterations in T_4 glucuronidation. Finally, measuring serum T_4 half-life would be a more direct measure of the adverse effect of increasing T_4 clearance.

4.2.6 Role of Binding Proteins

165. Another prevailing theory proposed to explain the mechanism by which some chemicals can reduce circulating levels of thyroid hormone is that of displacing the hormone from serum binding proteins – especially transthyretin in rodents (Brouwer et al. 1998a). This hypothesis is supported by the observation that certain biphenyls can displace T_4 from transthyretin with great affinity (Chauhan et al. 2000). Although provocative, TTR-null mice are euthyroid as are humans with a TTR deficiency (Palha 2002). Thus, it does not appear that TTR is a requirement for normal thyroid hormone homeostasis. However, it is likely to be important to measure serum binding proteins as a way of interpreting changes in serum total T_4/T_3.

166. In mammals, the serum-binding proteins for thyroid hormones are thyroid-binding globulin (TBG), transthyretin (TTR), and albumin (see review above). T_4 exhibits a greater affinity for TBG and TTR than does T_3 (*25*). Although TBG is present both in humans and rodents, the role of TBG in thyroid physiology in rodents is less well understood than in humans. However, TTR is present in humans, rodents, and nonhuman primates (Schussler 2000). In addition, there is speculation that xenobiotics can alter circulating levels of thyroid hormone by displacing T_4 from TTR (Brouwer et al. 1998b; Chauhan et al. 2000). Although this hypothesis is plausible, it is by no means proven. Thus, effects of xenobiotics on serum protein binding are not known to produce adverse effects.

167. It has also been suggested that xenobiotic binding to TTR is predictive of interactions with other T_4 binding proteins such as the deiodinases and sulfotransferases as well as chemicals with potential for high fetal accumulation (Brouwer et al. 1998b). These assays have been performed in several laboratories examining xenobiotics for several decades [e.g., (Brouwer and van den Berg 1986)]. Although these assays can be modified for high throughput screening, they are specific for chemicals that compete with ^{125}I-T_4 for serum binding proteins and will not detect chemicals that act through other mechanisms. In addition, the use of either TBG or TTR may not be relevant for nonmammalian species such as teleosts. However, one of the strengths of this assay is that it may be predictive of chemicals that alter fetal concentrations of TH and may provide for a useful screen in this capacity.

168. No comprehensive examination of the ability of individual toxicants or their metabolites to interfere with TH signaling has been reported. However, several recent reports have begun to characterize the ability of various xenobiotics to bind to the TR and/or to affect TH signaling *in vitro* and *in vivo.* Bogazzi et al. (2003) reported that the PCB mixture Aroclor 1254 can displace T_3 from rat TRβ1 at 10 μM. Ten micromolar Aroclor also inhibited T_3-induced CAT activity from a DR4-tk-CAT reporter construct in COS-7 cells co-transfected with the rat TRβ1 receptor in their study, indicating that individual congeners, or the mixture, can act as TRβ1 antagonists. They did not observe that Aroclor affected the ability of the TR to bind to a TH response element (TRE) using electrophoretic mobility shift assay (EMSA), but they did report that in the presence of Aroclor, the TRβ1 protein is more sensitive to protease digestion than in the presence of T_3, indicating that Aroclor binds to the TRβ1 protein and affects its conformation differently than does T_3.

169. These findings of Bogazzi et al. conflict to some degree with those of Koibuchi's group (Iwasaki et al. 2002; Miyazaki et al. 2004). Specifically, Iwasaki et al. (2002) reported that a specific hydroxylated PCB (4-OH-CB106) inhibited relative luciferase activity driven by a DR4-tk-Luc reporter in the presence of TRβ1 and T_3 in CV-1, 293, and TE671 cells. This inhibition occurred at a very low dose (10^{-10} M).

Miyazaki et al. (2004) followed this observation with an experiment indicating that 4-OH-CB106 causes the TR to become partially dissociated from the DR4 TRE using EMSA. Differences in study design between the Bogazzi et al. and the Koibuchi et al. reports (e.g., the use of Aroclor by Bogazzi et al. and 4-OH-PCB106 by Koibuchi's group) do not clearly reconcile their unconcordant results. An important issue may be the use of parent PCBs versus an hydroxylated metabolite.

170. Kitamura et al. (2005) have surveyed a large number of hydroxylated PCBs, finding nine that bind to the TR. However, in GH3 cells, all of these nine hydroxylated PCBs acted as TR agonists, increasing GH production and GH3 cell growth. In Kitamura's work, the source of TR for their binding studies was nuclear extracts from MtT/E-2 cells, so it was not possible to discriminate between binding to TRα and TRβ. Moreover, no parent PCBs tested singly exhibited TH-like effects in GH3 cells. Finally, none of the compounds that exhibited TH-like effects in Kitamura's experiments were found to exert estrogenic activity in MCF-7 cells; thus, there was a clear separation of thyroid hormone and estrogenic activities.

171. Arulmozhiraja and Morita (2004) studied the structure-activity relationships of 24 different hydroxylated PCBs on thyroid hormone "activity" in a yeast-two hybrid system. In this system, the ligand-binding domain of the human TRα receptor was linked to the coactivator TIF2 and inserted into the S. cervisiae Y190 cells that carried the β-galactosidase reporter gene. All of the 24 hydroxylated PCBs evaluated were low in chlorine number (tetra-, tri-, and di-chloro). The most active ligand was 6-OH-CB49 with 3.7% of T_3 activity on the ligand binding domain of the human TRα1.

172. Best characterized as a weak estrogen (Staples et al. 1998), bisphenol A (BPA) binds to the estrogen receptor (ER) with a Ki of approximately 10^{-5}M (Krishnan et al. 1993; Gaido et al. 1997). However, BPA binds to and antagonizes T_3 activation of the TR (Kitamura et al. 2002; Moriyama et al. 2002a) with a Ki of approximately 10^{-4} M, but as little as 10^{-6} M. At these concentrations, BPA significantly inhibits TR-mediated gene activation (Moriyama et al., 2002a). Additionally, Moriyama et al. found that BPA reduced T_3-mediated gene expression in culture by enhancing the interaction with the co-repressor N-CoR (Moriyama et al. 2002b). Developmental exposure of BPA in rats produces an endocrine profile similar to that observed in thyroid resistance syndrome (Cheng, 2005). Specifically, T_4 levels were elevated during development in the pups of BPA-treated animals, but TSH levels were not different from controls (Zoeller et al. 2005). This profile is consistent with BPA inhibition of TRβ-mediated negative feedback. However, the thyroid hormone-response gene RC3 was elevated in the dentate gyrus of these BPA-treated animals (Zoeller et al. 2005). Because the TRα isoform is uniquely expressed in the dentate gyrus, the conclusion was that BPA may be a selective TRβ antagonist *in vivo*.

173. If BPA acts as a TR antagonist *in vivo,* it is predictable that specific developmental events and behaviors would be affected by developmental exposure to BPA. In this regard, Seiwa et al. (2004) have shown that BPA blocks T_3-induced oligodendrocyte development from precursor cells (OPCs). In addition, there may be an association between the thyroid resistance syndrome and attention deficit-hyperactivity disorder (ADHD) in humans (Hauser et al. 1998; Vermiglio et al. 2004; Siesser et al. 2005) and in rats (Siesser et al. 2005); therefore, it is potentially important that BPA-exposed rats exhibit ADHD-like symptoms (Ishido et al. 2004).

174. Despite the antagonistic effects of BPA on the TRβ, halogenated BPAs may act as TR agonists (Kitamura et al. 2002). Both tetrabromo- and tetrachlorobisphenol A (TBBPA and TCBPA, respectively) can bind to the thyroid hormone receptor and induce GH3 cell proliferation and growth hormone production (Kitamura et al. 2002). In contrast, Ghisari et al. (2005) found that both BPA and its halogenated derivatives increased GH3 cell proliferation. Thus, these compounds may exert agonistic effects on the TR and this could be important during early brain development. For example, thyroid hormone of maternal origin can regulate gene expression in the fetal brain (Dowling et al. 2000; Dowling

and Zoeller 2000; Dowling et al. 2001); one of these genes codes for Hes1 (Bansal et al. 2005). Considering the role of HES proteins in fate specification in the early cortex (Gaiano and Fishell 2002; Schuurmans and Guillemot 2002; Wu et al. 2003), the observation that industrial chemicals can activate the TR and increase HES expression (Bansal et al. 2005) may indicate that these chemicals can exert effects on early differentiative events.

4.2.7. Incorporating Thyroid Endpoints into Pre-existing Assays

175. To date, all known thyroid toxicants in mammals have been identified by their ability to change measures of thyroid function, specifically circulating levels of thyroid hormone and changes in thyroid weight and histology (Brucker-Davis 1998). Based on this observation, DeVito et al. (1999) recommended that serum T_4 be used as a central feature of screening and testing programs for thyroid toxicity with concurrent evaluation of thyroid histology to support this screen. Pre-existing mammalian *in vivo* assays are designed to accomplish specific goals, whether it be to evaluate developmental toxicity or reproductive toxicity. Therefore, endpoints of thyroid function can be incorporated into these pre-existing assays, but the timing and duration of toxicant exposure and timing of endpoint acquisition must be carefully designed considering the information presented as background in this DRP. In addition, subchronic and long-term (chronic toxicity and/or carcinogenicity) studies may also provide evidence of thyroid toxicity. In particular, these regularly required tests to screen pesticides will provide relevant information about whether a given compound is a thyroid toxicant.

4.3 Current *in vivo* Rodent Screens

176. This section focuses on the existing *in vivo* mammalian assays developed by efforts within the OECD, Japan, and the USEPA.

177. The OECD *in vivo* mammalian assays include the following designs in rats:

1. OECD Test Guideline 407: Repeated Dose 28-Day Oral Toxicity Study
2. OECD Test Guideline 414: Prenatal Developmental Toxicity Study
3. OECD Test Guidelines 415/416: One- and Two-Generation Reproductive Toxicity Studies
4. OECD Test Guidelines 421/422: Reproduction/Developmental Toxicity Screening Test and Combined Repeated Dose Toxicity Study with the Reproduction/Developmental Toxicity Screening Test.
5. OECD Draft Test Guideline 426: Developmental Neurotoxicity Study.

178. Japanese researchers are developing computer-based screening models, *in vitro* cell lines, and a "one life-span test" in rodents.

179. The current *in vivo* mammalian assays developed by efforts within the USEPA include the following designs in rats:

1. One-generation study (also see OECD 415)
2. Two-generation study (also see OECD 416)
3. 20-day pubertal female study

4. 20-day pubertal male study

5. 15-day adult male study.

The EPA's Office of Pesticide Programs has issued "Guidance for Thyroid Assays in Pregnant Animals, Fetuses and Postnatal Animals, and Adult Animals," which recommends when/how the endpoints described in the assays listed above should be collected depending on the assay and chemical being studied.

180. The study designs for the assays listed above vary as will be described below, but in every case, the endpoints included were originally designed to capture measures of reproductive or general toxicity. Thus, the goal of this section is to demonstrate how the addition of endpoints that will capture thyroid toxicity can be included. Specific endpoints will be discussed below. These endpoints need not be added to all assays; rather, strategic additions should be made to include a combination of developmental and adult thyroid endpoints.

4.3.1 Endpoints for Thyroid Toxicity in Rodent Developmental Assays

181. The current endpoints proposed for thyroid toxicity in the *in vivo* OECD and USEPA mammalian assays listed above include thyroid weight and histopathology, as well as hormone measurements (T_4 and TSH, and perhaps T_3). Thyroid weight provides a measure of its stimulation by TSH over time; thus, if thyroid hormone levels are altered slightly for some duration, thyroid weight may reflect this change before technical assays can detect changes in serum hormone levels. Likewise, thyroid histopathology may provide a more sensitive indicator of this process and may be interpreted as a potential cancer endpoint. McNabb et al. (2004) recently employed a thyroid endpoint that may be more sensitive and simpler to recruit than thyroid weight and histopathology, which should be considered for development and possibly validation. Specifically, they measured the T_4 content of the thyroid gland in response to ammonium perchlorate exposure in bobwhite quail and found that this measure was far more sensitive to perchlorate exposure than was either serum T_4 concentration or thyroid weight (McNabb et al. 2004). Although this measure cannot be taken to indicate cancer, this measure (intra-thyroidal T_4) may be an important and easily captured endpoint for thyroid toxicity.

182. It is important to recognize that changes in circulating levels of thyroid hormones (and thyroid histology) represent important precursor events to adverse outcomes. This issue has been addressed most clearly by Crofton (Crofton 2004; Crofton et al. 2005). In a seminal series of studies, Crofton found a linear relationship between PCB-induced reductions in circulating levels of thyroid hormone and adverse outcome (i.e., hearing) (Crofton 2004; Crofton et al. 2005). Thus, at least for one thyroid hormone effect, the relationship between hormone level and hormone action has been characterized (Crofton and Zoeller 2005).

183. The endpoints of thyroid gland weight and histology, serum T_4, T_3, TSH, and, potentially, intra-thyroidal T_4 are measures of thyroid function, which are and should remain key endpoints of thyroid toxicity studies. Importantly, not all toxicants that cause a reduction in serum thyroid hormone levels produce the same profile of hormonal and thyroid changes. For example, PTU exposure produces a decrease in circulating levels of T_4, T_3, and a concomitant increase in circulating levels of TSH (Zoeller 2005). In contrast, PCB exposure causes a decrease in serum T_4, but this is not usually associated with an increase in serum TSH (Zoeller 2005). It is possible, though not well studied, that the specific profile of hormonal changes represents a "fingerprint" for the mechanism of action that mediates the antithyroid effect. In the example above, PTU is a TPO inhibitor and PCBs likely reduce serum T_4 by inducing UDPGTs, and perhaps by displacing T_4 from serum binding proteins.

184. However, regardless of the mechanism by which circulating levels of thyroid hormone are reduced, it may be important to determine how a decrement in serum thyroid hormones specifically affects tissues that require thyroid hormone (especially in development).

185. Therefore, to resolve whether a thyroid toxicant affects brain development, for example, measures of thyroid hormone action in the developing brain would be informative. Similar logic would be applied to resolve whether a thyroid toxicant affects heart or lung development, or adult physiological functions. Recommended rodent developmental endpoints known to be sensitive to thyroid hormone insufficiency are discussed later in this chapter in the context of potential inclusion in existing *in vivo* screens and tests. These endpoints may be considered to be measures of specific effects as well as generalized endpoints of thyroid disruption. From this point of view, endpoints of thyroid hormone action in non-mammalian vertebrates (e.g., frog metamorphosis) may also be considered to be generalized endpoints of thyroid disruption. The concept that one endpoint of TH disruption could be emblematic of TH disruption in general is complex and requires additional information. For example, the ability to metabolize xenobiotics is different among the vertebrate taxa and this may affect the generalizability of specific endpoints of TH action. In addition, there may be sufficient difference among vertebrates in the various proteins that govern the thyroid system (e.g., receptors, peroxidases, transport proteins, etc.) that xenobiotics will not interact with all of these proteins in the same way across the vertebrates.

186. The assays proposed for screening and testing for endocrine disruptors are reviewed below, and exposure times and endpoints are described to familiarize the reader with current thyroid analyses. This section will help the reader visualize how well new endpoints or assays may fit into or alter the current rodent methods for analysis of the thyroid system.

187. Current ways of detecting thyroid toxicity include measures of hormone levels by radioimmunoassay, characterization of thyroid function using histopathological techniques, and in some cases computer assisted morphometry. Although radioimmunoassays are commonly used in current assays, there should be some standardization of the kits used or the analysis methods employed. Some groups evaluate RIAs by extrapolating from the lowest standard to the "zero" tube because some or many of their samples are below the lowest standard, possibly leading to inaccurate measures of hormone levels. This situation likely arises in part because total T_4 in rat serum runs near the low end of the standard curve that is calibrated for humans. Direct endpoints of toxicant effects on thyroid hormone action would require validated methods of measurement that could be calibrated across labs. Real-time PCR techniques would likely provide the greatest degree of quantitative reliability because a standard curve could be generated that would provide a basis for comparison across labs. However, if endpoints of thyroid hormone action were to be included in screens and tests for thyroid toxicants, techniques required to capture these endpoints (*in situ* hybridization, northern blots, etc.) would have to be in some way standardized. While many gene expression changes are being developed as potential endpoints for screens, these endpoints are still in the research and development phase and most are not yet ready for validation.

4.3.2 OECD Test Guidelines

188. Thyroid endpoints for the OECD Test Guidelines can be proposed as additional endpoints to add on to the existing assay protocols. The thyroid endpoints currently under consideration for the OECD Test Guidelines are the same as those in the EDSP—namely, thyroid weight, hormone analysis (T_4, T_3, TSH), and thyroid gland histopathology. In the material presented below, the term "screening assay" refers to a protocol designed to obtain initial information about the ability of a compound to interfere with the thyroid system. The goal of this test is to maximize the number of true positives and minimize the number of false negatives. In contrast, the term "test" or "testing assay" would establish if a substance could cause effects through the thyroid system, determine the consequences to the organism studied, and establish a dose response relationship between the substance and the effects observed in the test.

4.3.2.1 OECD TG 407 - Repeated Dose 28-day Oral Toxicity Study in Rodents

189. This is a 28-day assay to evaluate a test chemical's oral toxicity using repeated daily doses in adult animals. The preferred rodent species is the rat, although other rodent species may be used. Females should be nulliparous and non-pregnant; dosing should begin as soon as possible after weaning and, in any case, before the animals are 9 weeks old. The route of administration should be by gavage, dosed feed, or dosed water. This study will indicate the potential health hazards of a test chemical after repeated exposure for a relatively short duration, especially immunological and neurological effects as well as reproductive toxicity. The TG 407 protocol was recently enhanced to include the thyroid endpoints mentioned above. This assay is considered to identify a test chemical's effects through clinical observation, hematology, clinical biochemistry of the blood serum and urine, pathology, and histology on organs that are chosen according to the user's needs. Results from this assay will inform the chemical testing community on how to proceed with further tests.

4.3.2.2 OECD TG 414 - Prenatal Developmental Toxicity Study

190. OECD TG 414 tests for the effects of prenatal toxicant exposure (normally by intubation) on both the pregnant test animal and the developing offspring. Animals are dosed with the test chemical from implantation (around 5 days after mating) to 1 day before the planned caesarean section. This test will usually include the entire period of gestation, but can be shortened depending on the needs of the administering scientist. The assay is designed to observe effects on organogenesis. Suggested endpoints include: clinical observations; analysis of the dams including a complete examination of the uterus; and analysis of the fetus including sex, external alterations, and skeletal and soft tissues analysis. No specific thyroid endpoints are in included in this assay. This assay corresponds to U.S. EPA's Developmental Toxicity Assay and the U.S. Food and Drug Administration's (USFDA's) Segment II study.

4.3.2.3 OECD TG 415 - One-Generation Reproduction Toxicity Study

191. OECD TG 415 tests for a chemical's effects on male and female reproductive performance (i.e., gonadal function, estrus cyclicity, mating behavior, conception, parturition, lactation, and weaning). The One-Generation assay also identifies developmental toxicity (i.e., neonatal morbidity, mortality, behavioral abnormalities, teratogenesis). It corresponds to the EPA's One-Generation Assay, but doses animals earlier than the EDSP's proposed One-Generation Reproduction assay.

192. The experimental schedule for this assay doses the parental generation prior to mating (at least 10 weeks for male rats and 2 weeks for female rats) and then throughout mating. The dams are then dosed throughout gestation and lactation until weaning of the F_1 generation. Dosing and necropsy of the F1 generation are adjusted according to the intended use for this assay (see EDSP section on the One-Generation assay below). The endpoints included in the test guideline include physical observations, and histopathology of the ovaries, uterus, cervix, vagina, testes, epididymides, seminal vesicles, prostate, coagulating gland, and the pituitary gland. Other target organs may be added as necessary. Thyroid endpoints, including those mentioned above, could easily be added to this assay.

4.3.2.4 OECD TG 416 - Two-Generation Reproductive Toxicity Studies

193. The OECD's TG 416 corresponds to the EPA's Two-Generation Reproductive Toxicity Test as described below. The EDSP's proposed Two-Generation Test differs from TG 416 in that the dosing does not begin prior to mating, whereas the TG 416 begins dosing the male rats at least 10 weeks prior to mating and the female rats at least 2 weeks prior to mating. In both guidelines, the dosing begins with the parental generation, continuing throughout mating, pregnancy, and lactation to weaning of the F1 generation. The F1 offspring, once weaned, are dosed throughout development, mating, pregnancy, and lactation, to

weaning of the F2 generation. Results from this assay are used to assess whether additional studies are required.

194. Endpoints outlined for this assay are very similar to those described for the One-Generation assay (TG 415), but also include estrus cycle and sperm evaluations, extensive observation of the offspring in the F1 and F2 generations, and organ weights of dosed animals (uterus, ovaries, testes, epididymides, prostate, seminal vesicles and coagulating glands and fluids, brain, liver, kidneys, spleen, pituitary, thyroid, and adrenal glands). Other target organs can also be added on as needed. Histopathology of the parental and F1 generation are also required for certain organs (vagina, uterus with cervix, ovaries, 1 testis, 1 epididymis, seminal vesicles, prostate, and coagulating gland), and additional ones can be examined if necessary.

4.3.2.5 OECD TG 421 and 422 - The Reproduction/Developmental Toxicity Screening Test and the Combined Repeated Dose Toxicity Study with the Reproduction/ Developmental Toxicity Screening Test

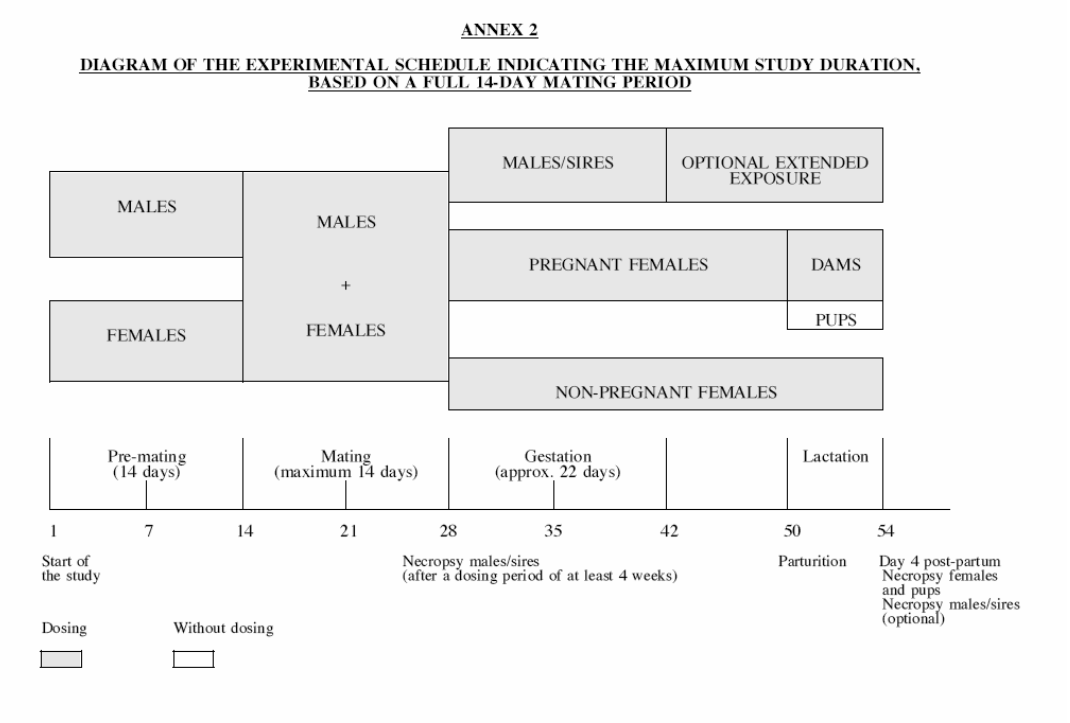
195. OECD TG 421 and 422 are both screening assays designed to provide the initial information on the effects of a test chemical on male and female reproduction (Figure 4-2). Both screens offer limited information on whether a test substance causes abnormal postnatal effects after prenatal exposure, or if the effects are due to postnatal exposure. Because these are both considered screens, negative data do not indicate that a chemical is safe. TG 422 also focuses on neurological endpoints.

196. Exposure schedules for these test guidelines are approximately 54 days long with dosing for ~14 days pre-mating, ~14 days mating (or less), 22 days during gestation, and then 4 days of lactation.

197. Endpoints for this assay include clinical observations of adults, body weight, and food consumption changes throughout the study, pathology, and histology (for reproductive organs and accessory sex glands). TG 422 also includes hematology, clinical biochemistry on blood plasma or serum samples and urine. Histopathology includes organs other than the reproductive organs such as the brain (cerebellum, cerebrum, pons), spinal cord, stomach, small and large intestines, liver, kidneys, adrenals, spleen, heart, thymus, thyroid, trachea, lungs, uterus, urinary bladder, lymph nodes, peripheral nerve, and bone marrow. Pups are observed after necropsy for external gross abnormalities. Thyroid hormone serum analysis and thyroid histopathology are included as potential endpoints for these two test guidelines.

198. No currently proposed EDSP assay is similar to these two test guidelines in the dosing schedule or the proposed endpoints.

Figure 4-2 Study Design for OECD Test Guideline 421 (Reprinted by Permission of OECD)



4.3.2.6 OECD Test Guideline 426: Developmental Neurotoxicity Study (Draft 2003).

199. This test guideline was initially developed based on the U.S. EPA's guideline for developmental neurotoxicity testing. OECD Expert Consultation Meetings were held in 1998 and in 2000 to develop this draft. The protocol is designed to be performed as an independent study. However, observations and measurements could also be incorporated into a perinatal developmental toxicity study, or added on to a one- or two-generation study. Thyroid endpoints could be added on to Tg 426, or to a fusion of Tg 426 and a one- or two-generation study. The recommendation is that dams be exposed to at least 3 doses of compound from gestational day (G) 6 to weaning at PND 21. Pre-weaning measures would occur on PND 22 and would include behavioural ontogeny, brain weight, neuropathology and morphometry (optional). Post-weaning investigations would occur around PND 60 and would include behavioral/functional tests, motor activity, sexual maturation, auditory startle, brain weight, neuropathology and morphometry (optional).

4.3.2.7 Summary of OECD Test Guideline Thyroid Endpoints

200. The thyroid endpoints being considered as "add-ons" for these test protocols include thyroid gland weight, histology, and serum thyroid hormone measurements including TSH. As discussed previously, these endpoints can provide information about whether a chemical alters serum thyroid hormone levels. For example, endpoints of developmental neurotoxicity that are specific to thyroid hormone action may be considered to provide information that is important in interpreting the consequences of changes in thyroid hormone that were identified either by radioimmunoassay or by changes in thyroid weight/histopathology.

4.3.3 Japanese Screening and Testing Program

201. Overall, the screening and testing program for EDCs being developed by Japanese researchers is comprised of three components: 1) *In silico* screening using a 3D-SAR docking model (but not for TR), 2) *In vitro* assays using mammalian cell lines including a TR expression system, and 3) several *in vivo* assays for estrogen and androgen. A "Rodent one life-span test" that will include endpoints for thyroid toxicants is also being developed as the Tier 2 "definitive" rodent test. The presumptive one life-span test protocol would monitor the major stages of one life-span of rodents, including conception, *in utero* development, growth, maturation, and senescence. The exposure period may be perinatal and the monitoring periods would be not only around puberty but also in adulthood and/or early senescence. Currently, the endpoints under consideration will cover reproductive endpoints as well as endpoints for neurotoxicity and the immune system, with an emphasis on functional endpoints including acceleration of senescence-related phenotypes. Toxicogenomics approaches may be incorporated for monitoring the molecular events underlying the adverse effects. It will be important to incorporate endpoints of thyroid toxicants in this one life-cycle test.

4.3.4 U.S. EPA's EDSP Rodent Assays

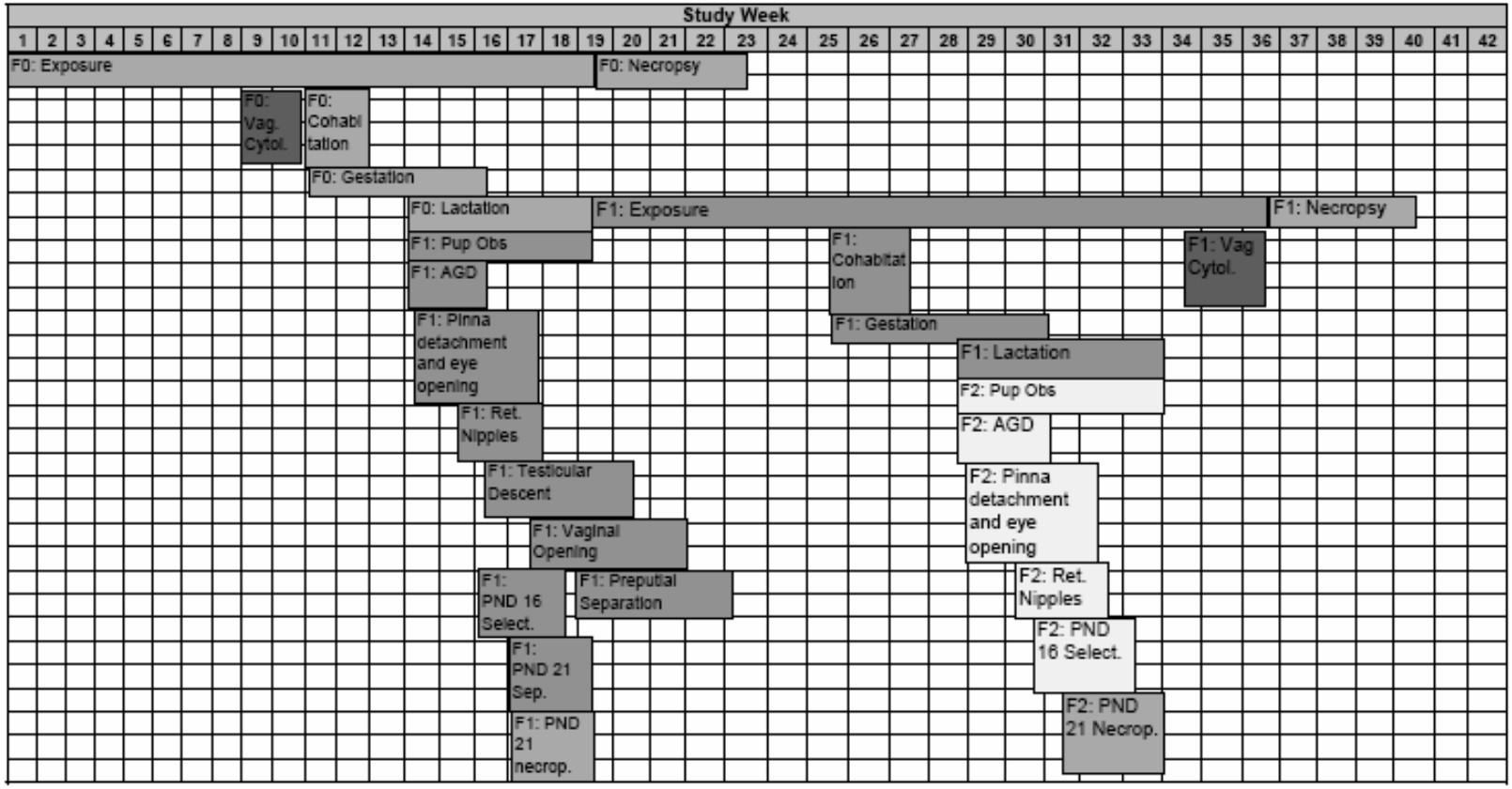
202. As with the OECD test guidelines, the proposed assays in the EDSP battery contain thyroid endpoints that were added to assays for reproductive and developmental toxicity. These thyroid endpoints, generally proposed as "add-ons" in the EDSP battery of assays, are thyroid gland weight and histology, and serum thyroid hormone measures (T_3, T_4, TSH).

4.3.4.1 Two-Generation Study (Similar to OECD TG 416)

203. One of the tests being considered for inclusion in the EDSP is a rat, two-generation reproductive toxicity test that could be modified for thyroid toxicity. It is similar to the OECD TG 416. The basic two-generation test is described by the EPA Office of Prevention, Pesticides, and Toxic Substances' Health Effects Test Guideline 870.3800: Reproduction and Fertility Effects (U.S. EPA 1998): http://www.epa.gov/scipoly/oscpendo/docs/edmvs/ptu2gendraftforedmvs.pdf. The assay is illustrated in Figure 4-3.

204. Thyroid endpoints under consideration for this test protocol include thyroid weight, histology, and thyroid hormone analysis of T_4 and TSH. This test has completed pre-validation with the thyroid endpoints included. The two-generation assay is a Tier 2 test that identifies functional disruption of the estrogen, androgen, and thyroid systems during exposure to a chemical over two generations.

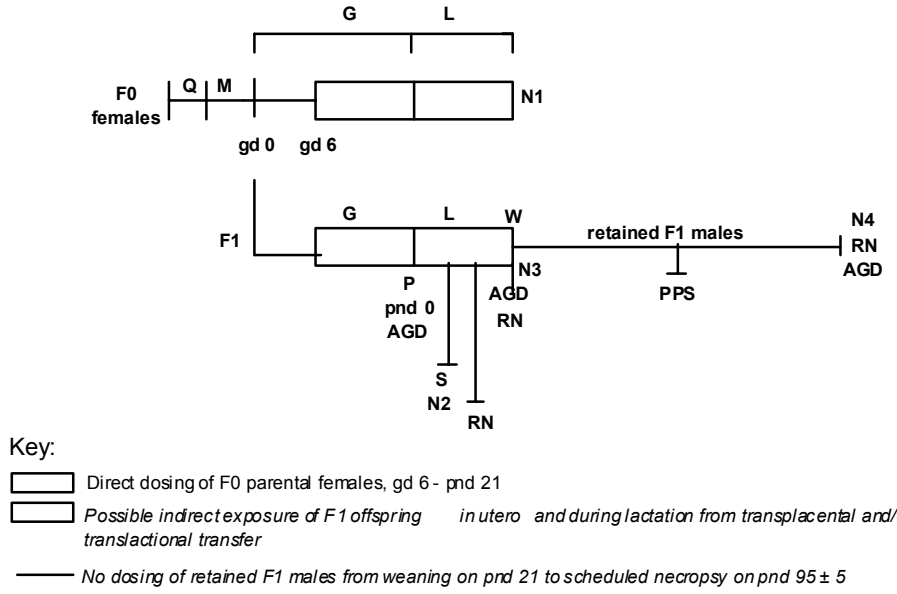
Figure 4-3 Exposure and Endpoint Collection in EPA's Two-generation Reproductive Toxicity Assay



4.3.4.2 One-Generation Assay (Similar to OECD TG 415)

205. Although the basic two-generation study design was developed to provide information on insult to the reproductive tract, there is concern that certain effects may be missed because the reproductive tract has not had sufficient time to develop before the observations are made. In the standard two-generation test, most F1 animals are sacrificed and examined at PND 21; only one animal per sex per litter is usually allowed to continue to maturity, and these animals are then used to breed the F2 generation. An alternative to the two-generation study is a one-generation study that would allow for examination of the F1 males past puberty at pnd 90 +/-2. The study design tests whether continuing toxicant exposure in the F1 generation males through puberty to adulthood will provide additional information for detection of endocrine-mediated effects. The one-generation study has been proposed as an alternative to the two-generation study. In addition, the USEPA's EDSP conducted a special study of a one-generation test that was added on as an extension to a two-generation assay and continued the F1 male generation out to pnd 95 +/-5 (Gray et al. 2003). It should be noted that the original study design described only utilized F1 males, but future assays will likely also include females. The study design is illustrated in Figure 4-4.

Figure 4-4 Study Design to Examine Effects in F1 Offspring



206. The objectives of the one-generation study and the one-generation extension study are to determine the following: 1) Can some of the effects of perinatal exposure to thyroid toxicants be missed if the timing of endpoint acquisition is structured to identify reproductive toxicants in post-weanling animals, and 2) Do some of these effects occur at an incidence that would go undetected if only one male per litter were retained past puberty and examined in adulthood?

207. Retaining a greater number of the F1 males to examine at or after puberty may allow for greater distinction of the thyroid endpoints such as thyroid growth and histology. It is not yet clear whether the EDSP will proceed in validating the basic one-generation study to use as an alternative to the two-generation assay or the one-generation extension of the two-generation study.

208. Endpoints of thyroid function are included in the one- and two-generation assays. As more endpoints are developed and incorporated into these regulatory assays, more specific effects of toxicants will be identified, decreasing the possibility that these regulatory assays will produce false negatives.

4.3.4.3 Female Pubertal Assays

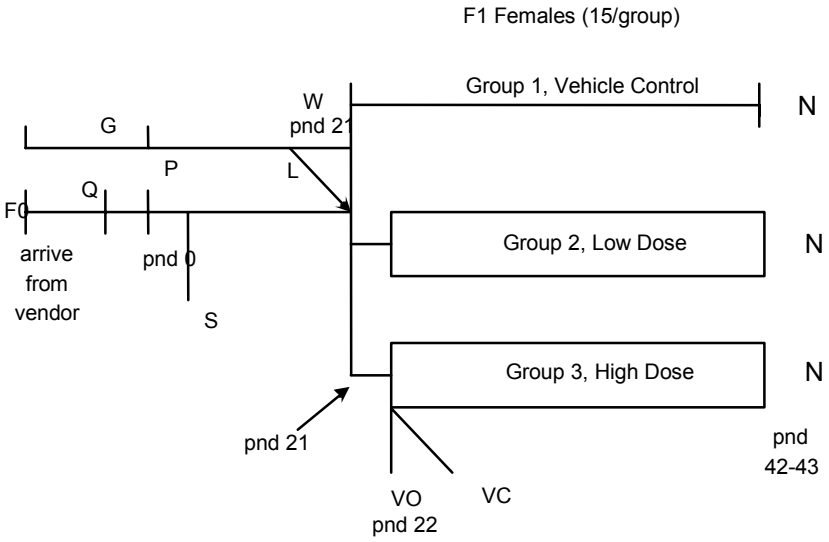
209. The EDSTAC, assembled by the EPA in 1996, recommended the use of a female 20-day pubertal assay with endpoints to evaluate test materials for effects on the thyroid, the hypothalamic-pituitary-gonadal (HPG) axis, and aromatases. The EPA, at the recommendation of the EDSTAC, has proposed to include a female pubertal assay in an endocrine disruptor screening program. This assay (Figure 4-5) is the most comprehensive assay in the proposed Tier 1 battery of assays, as it is capable of detecting substances that alter thyroid function, inhibit aromatase, act as estrogens or antiestrogens, and interfere with the hypothalamus-pituitary-gonad/thyroid axis (EDSTAC Report 1998, Vol. 1, Chapter 5, pp. 5-26 to 5-27). The female pubertal assay is currently being validated by several labs. The protocol for the female pubertal assay measures the following thyroid endpoints: serum T_4 and TSH concentrations, thyroid gland histology, thyroid gland weight, and body weight changes. Results from other, shorter assays and/or with the use of intraperitoneal (ip) injection as the route of administration, have also been reported (O'Connor et al. 1996, 1999). EDSTAC also recommended that the male 20-day pubertal assay in rodents (described in the next section) be evaluated as an alternate assay (EDSTAC 1998, Vol. 1, Chapter 5, p. 5-30; see Section 10.1.4.2).

210. In the female pubertal assay, toxicant exposure begins on the day of weaning (pnd 21). Thus, many of the developmental endpoints sensitive to thyroid hormone (see below in this chapter) have passed. Two potential endpoints of thyroid hormone to be considered during puberty and potentially in future versions of this assay protocol include measures of myelination and toxicant effects on BrdU-labeled cells in the hippocampus. Addition of these endpoints will require research and development, followed by protocol standardization and validation. Although this process is not immediate, it is important to consider new thyroid endpoints as the state of thyroid research expands, and in recognition that the current assay endpoints will need to be improved as more information on the thyroid system becomes available.

4.3.4.4 Male Pubertal Assay

211. The EDSTAC also recommended that a 20-day male pubertal assay in rodents be evaluated as an alternative assay (EDSTAC 1998, Vol. 1, Chapter 5, p. 5-30). This assay is the most comprehensive assay in the proposed Tier 1 battery of assays, as it is capable of detecting substances that alter thyroid function, inhibit aromatase, act as androgens or anti-androgens, and interfere with the hypothalamus-pituitary-gonadal axis (EDSTAC 1998, Vol. 1, Chapter 5, pp. 5-30 through 5-32). The study design for the male pubertal assay (Figure 4-6) is similar to the female pubertal assay. The male pubertal assay has been lengthened to a 30-day pubertal assay that covers PNDs 22-52 and is currently being validated. It includes the following thyroid-related endpoints: body weight, thyroid gland weight, thyroid gland histology, and T_4 and TSH plasma concentrations at necropsy. Therefore, the EDSP is pursuing the validation of a male pubertal assay as a potential alternative to other assays in the Tier 1 battery.

Figure 4-5 Female Pubertal Assay



KEY:

——— No test chemical exposures to F0 dams or F1 offspring during gestation or lactation, no exposure of F1 females in Group 1; F1 females dosed with corn oil.

▭ Direct once daily gavage dosing with test chemical of F1 females starting on pnd 22

Q = Quarantine (seven days, gd 13-20)

G = Gestation

P = Parturition (pnd 0)

L = Lactation

W = Wean (pnd 21) F1 pups

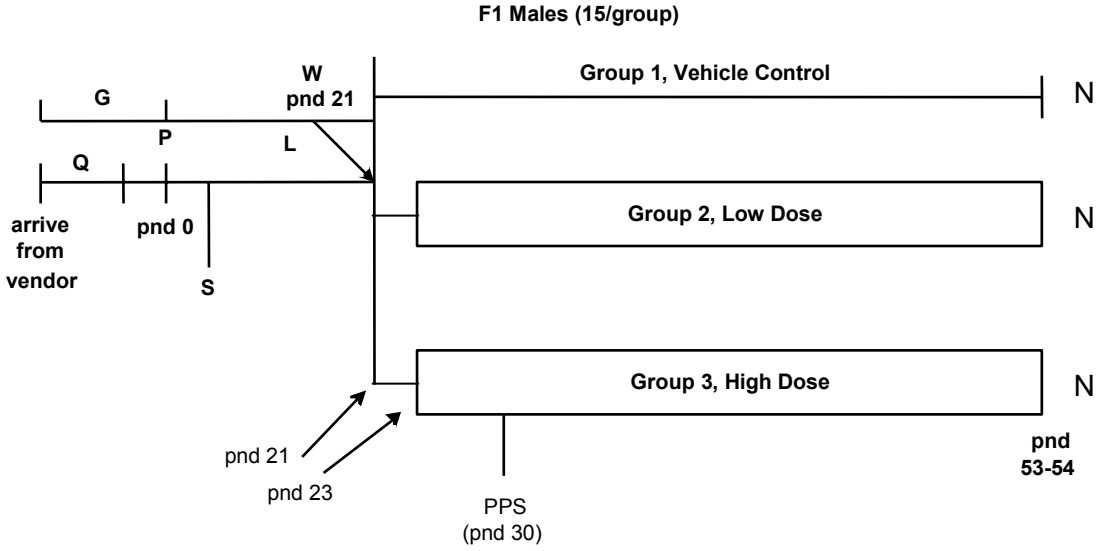
S = Standardize litters to eight to ten with maximum number of F1 female pups (discard culled pups)

VO = Acquisition of vaginal opening (evaluation begins on pnd 22)

VC = Vaginal cytology (evaluation begins on the day of VO)

N = Necropsy

Figure 4-6 Male Pubertal Assay



No exposures to F0 dams or F1 offspring during gestation or lactation

Direct once daily gavage dosing of F1 males beginning on pnd 23

Q = Quarantine

G = Gestation

P = Parturition (pnd 0)

L = Lactation

W = Wean (pnd 21) F1 pups

S = Standardize litters to eight to ten with maximum number of F1 male pups (discard culled pups)

PPS = Acquisition of preputial separation (evaluation begins on pnd 30)

N = Necropsy

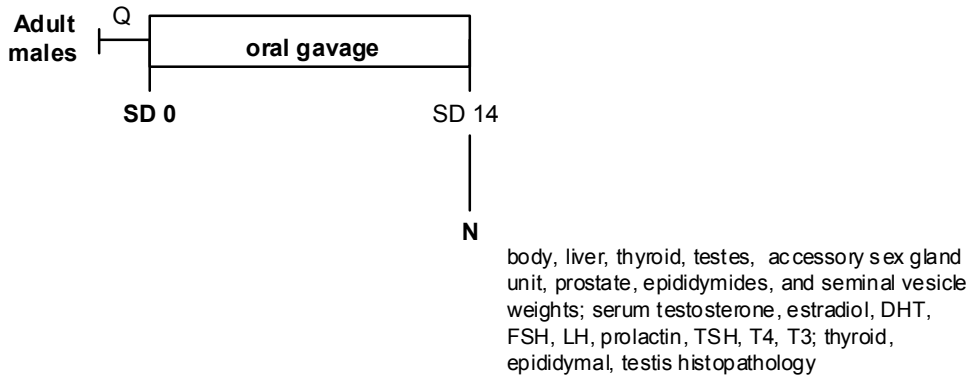
4.3.4.5 15-Day Adult Male Screen

212. One of the assays recommended by the EDSTAC as an alternate assay is a short-term screen in intact adult males. The adult male assay was developed to identify compounds that have the potential to act as agonists or antagonists to the estrogen, androgen, progesterone, or dopamine receptor. It can also identify 5α reductase inhibitors, steroid biosynthesis inhibitors, or compounds that alter thyroid function. Results from this assay and/or with the use of intraperitoneal injection as the route of administration, and other assays with a similar purpose, have been reported (O'Connor 1996, 1999, 2002a, 2002b).

213. The study design (Figure 4-7) is simple and straightforward: males are dosed once daily for 15 consecutive days (0 through 14). At necropsy on day 14, organ weights, histopathology, and circulating hormone levels are documented. These endpoints for thyroid hormone are the same as those described above. There are few obvious endpoints known to be specifically sensitive to thyroid hormone

insufficiency in adult animals within a 15 day exposure, which could be added into this assay in the future. However, possibilities include endpoints in the liver and heart (see below).

Figure 4-7 15-Day Adult Male Screen



4.3.5 Research and Development - Potential New Endpoints for Thyroid System Disruption

214. The existing *in vivo* screens are designed to identify agents that interfere with estrogen or androgen actions, and measurements of thyroid hormones and various aspects of thyroid gland morphology are "added on." Clearly, these are important additional endpoints to obtain because, without additional use of animals, and with careful consideration of the specific endpoints and the timing of their acquisition, valuable information can be obtained concerning toxicant effects on the thyroid system. Therefore, this section focuses on how existing protocols may be designed to provide strategic measures of thyroid toxicity, based on the background of information described in this DRP. An important consideration for evaluating potential thyroid toxicity is the timing of exposure, the specific endpoints evaluated, and the timing of acquisition of those endpoints.

215. Thyroid hormone exerts specific effects on development, but those effects are different in different tissues and at different developmental times. Therefore, the development of endpoints for thyroid toxicants will require selection of specific thyroid-specific endpoints and characterization of their sensitivity to both thyroid hormone insufficiency and to thyroid toxicants that are appropriate for the timing dictated by the developmental time at which endpoints are acquired. Unfortunately, endpoints of TH action, either during development or in adults, have not been examined extensively within the context of dose responses of thyroid hormone insufficiency. Therefore, it is not possible to make recommendations about the relative sensitivity of potential endpoints of thyroid toxicology without acknowledging the need to define this dose response relationahip and the fact that, despite the long-time recognition that thyroid hormone is essential for brain development and physiology, there are few thyroid-dependent endpoints sufficiently characterized that will be easily recruited for toxicological studies.

216. For example, a transient 30% decline in maternal TH, which is itself not associated with overt measures of toxicity such as a change in maternal or fetal body or brain weight, can significantly alter neuronal migration in the fetal cortex, creating a situation where the adult progeny have a large proportion of neurons that are found in ectopic locations within the cortex (Auso et al. 2004b). However, the TH insufficiency must occur during mid-gestation to have this effect. Post-natal exposure to TH insufficiency would not have this effect. Another example is that illustrated by Sui and Gilbert (2003) and Sui et al. (Sui et al. 2005), in which timed TH insufficiency can produce long-term effects on synaptic function in the hippocampus. These examples illustrate that the timing of TH insufficiency (or toxicant exposure) are critical determinants in a protocol designed to identify thyroid toxicants. This observation is consistent

with research in humans and in animals demonstrating that the timing of TH insufficiency determines the specific adverse effects (Zoeller and Rovet 2004).

217. Thus, the endpoints described below, many of which are still in the laboratory development phase, could be developed for inclusion in the test guideline protocols described above, and still remain as "add-ons." Many of the listed assays are highly specific indicators of chemicals for a narrow range of mechanisms. However, it is important to consider these assays until we have a better idea of the number of chemicals that act through the different pathways to disrupt the thyroid system.

4.3.6 Thyrotropin-Releasing Hormone (TRH) and Thyrotropin (TSH) Challenge Test

218. These assays test the functional integrity of the pituitary gland and thyroid gland respectively (Fail et al. 1999). Briefly, the TRH challenge test measures TSH concentrations before and after administration of purified TRH. Challenge with TRH should increase serum concentrations of TSH. A hyperreactive response is observed in the case of deficient thyroid function as the result of the lack of negative feedback on the pituitary gland. In contrast, a decreased TSH response to exogenous TRH is observed if the hypothalamus is deficient (Sarne and Refetoff 1995); (Fail et al. 1999). Although the TRH challenge has potential for providing mechanistic information on the actions of chemicals that alter thyroid function, the assay may not be a useful screen because of the limited number of chemicals that may act through this mechanism. Likewise, a TSH challenge could be employed to determine whether xenobiotic chemicals can affect thyroid gland sensitivity to TSH.

4.3.7 Thyroid Hormone Receptor Binding and Activation

219. In principle, chemicals could alter thyroid hormone signaling by binding to its receptor. There are several isoforms of the receptors that have tissue-specific localization (see the review above). Several environmentally relevant classes of chemicals have been proposed to bind to the TR, such as the polyhalogenated dioxins, dibenzofurans, biphenyls, and diphenyl *p* ethers (McKinney and Waller 1994, 1998; Porterfield and Hendry 1998; Porterfield 2000), but these proposals have not been adequately tested in the laboratory. Interestingly, a number of recent studies have begun to examine the ability of environmental chemicals to affect TR activation. Specifically, Zoeller et al. (Zoeller et al. 2000) reported that developmental exposure to PCBs can produce thyroid hormone-like effects on the expression of specific genes in the developing brain. However, they did not find that PCBs could displace T_3 from nuclear TRs (Gauger et al. 2004b). However, Miyazaki et al. (Miyazaki et al. 2004) reported that at least one hydroxylated PCB congener can cause the TR to dissociate from DNA. This is an important observation because it implies that the PCB congener is binding to an allosteric binding site on the TR that regulates its ability to interact with the gene's promoter. The observations of Zoeller and of Miyazaki are compatible considering that the unliganded TR is a constitutive repressor. Thus, PCB might increase the expression of RC3/Neurogranin by causing de-repression rather than T_3-activation. Others have shown that bisphenol A and its halogenated derivatives can bind to the TR and exert effects on TR-regulated gene expression (Kitamura et al. 2002; Moriyama et al. 2002). Finally, a new report indicates that 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) can augment T_3-induced gene expression in a cell line (Yamada-Okabe et al. 2004). These studies reveal that a variety of environmental chemicals can directly affect TR activation, perhaps in novel ways. It would be predictable that chemicals interfering with TR action should alter thyroid hormone levels in serum. For example, BPA binds to the TR and acts as an antagonist (Moriyama et al. 2002), and treatment of rats with BPA can increase serum T_4 (Zoeller, unpublished). However, this may not always be the case, especially if chemicals interfere with the TRα receptor because it does not mediate negative feedback on the pituitary.

4.3.8 The Developing Rodent Cerebellum

220. The effect of thyroid hormone insufficiency on brain development is dependent upon the timing of the insufficiency and the brain area examined. Thus, from a toxicological point of view, assays must be developed that specify the endpoint very clearly and whether this endpoint is mediated by TRα or TRβ (or both) receptors. Because the cerebellum has been so extensively evaluated for its developmental dependence on thyroid hormone (Koibuchi and Chin 2000b), this section focuses exclusively on this tissue.

4.3.8.1 Overview of Methods

221. Cerebellar granule cells originate in the external granule layer (EGL) and migrate to the internal granule layer (IGL) within the first 2 to 3 weeks after birth in the rat (Altman 1982; Altman and Bayer 1985). After their migration, a significant proportion of these cells undergo apoptosis by a Bcl-2-mediated pathway. A number of investigators show that thyroid hormone affects cerebellar granule proliferation, migration, and apoptosis (Muller et al. 1995; Xiao and Nikodem 1998a; Pasquini et al. 2000; Singh et al. 2003b). The role of thyroid hormone in the control of these developmental events is discussed below. These include the following:

222. *Cell proliferation*: This was originally performed using tritiated thymidine (^{3}H-thymidine). This building block of DNA is incorporated into the newly synthesized DNA of dividing cells and can be detected by autoradiography (Nicholson and Altman 1972b). This method requires the use of radioactivity and because it is a very weak beta-emitter, ^{3}H requires some considerable time to detect it in liquid emulsion. Another method is the use of Bromodeoxyuridine (BrdU). BrdU is a thymidine analogue that is incorporated into newly synthesized DNA by cells in S-phase (Menezes and Luskin 1994; Doetsch et al. 1997; Luskin et al. 1997). BrdU-labeled cells are detected using immunocytochemistry with an antibody available commercially that binds to DNA with BrdU incorporated into it. Using BrdU can allow one to detect the timing of cell birth to within a 1-hour period (e.g., (Wood et al. 1992). Finally, cell proliferation has been examined in the developing brain using an antibody to proliferating cell nuclear antigen (PCNA). PCNA antisera are available commercially and stain for a cyclin D2 that is present only in proliferating cells (Gobetto et al. 1995; Tanaka and Marunouchi 1998). This method has been used to identify proliferating cells in the developing cerebellum, and considering that the gene coding for PCNA has been cloned (Matsumoto et al. 1987), this can be used for *in situ* hybridization and dual-labeling if needed.

223. *Apoptosis*. The presence of DNA fragmentation identified by TUNEL staining (terminal deoxyribonucleotidyl transferse (TdT)-mediated biotin-16-dUTP nick-end labeling) is a late marker of apoptosis (Valavanis et al. 2001). In addition, there are several reports that TUNEL does not discriminate between programmed cell death and necrosis (Charriaut-Marlangue and Ben-Ari 1995; Grasl-Kraupp et al. 1995; Wullner et al. 1999). Therefore, TUNEL staining is often followed by a marker of early onset of apoptosis using immunocytochemical staining for activated caspase-3 (Valavanis et al. 2001). In addition, Singh et al. (2003b) have recently reported that the hypothyroidism-induced increase in apoptosis in the IGL is associated with down-regulation of Bcl-2 and Bcl-X_L expression, and up-regulation of Bax expression. Therefore, to support conclusions based on TUNEL and activated caspase-3 staining, it is also important to measure Bcl-2.

224. *Migration.* There is no specific biochemical marker of cell migration in the nervous system. However, this issue has been inferred by two types of methods. First, those studying TH effects on cell migration in the cerebellum have examined the number of cells in the mitral layer as well as their spindle shape (Morte et al. 2002a) as a measure of migrating neurons (from EGL to IGL). Second, those studying cell migration in the cerebral cortex have examined the position of cells within the cortical laminae. Neurons that occupy different layers in the adult cortex are born at different times, between gestational day (GD) 13 and GD20 (Takahashi et al. 1992; Caviness et al. 1995; Chenn et al. 1997). Recently, Lavado-

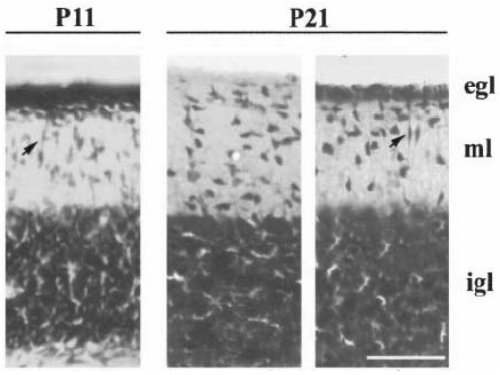
Autric et al. (Lavado-Autric et al. 2003) showed that subtle thyroid hormone insufficiency in rat dams can alter the migratory behavior of cortical neurons labeled on different days with BrdU. This resulted in a breakdown in the establishment of specific cortical layers.

4.3.8.2 Potential Endpoints of Cerebellar Granule Cells

225. During cerebellar development, large-scale cell proliferation occurs in cells of the external granule layer (EGL) during the first 3 weeks following birth. When proliferation within a specific lineage ends, cells begin to differentiate and migrate inwards to the internal granule layer (IGL). In normal rats, mitotic activity in the EGL declines with age and the EGL disappears at about 24 days (Nicholson and Altman 1972a). In hypothyroid animals there is a delay in granule cell migration and a persistence of the EGL. Lewis et al. found that in normal animals the EGL at 21 to 22 days of age was only one cell thick, whereas in hypothyroid animals, the EGL, persisted and at 35 days of age was finally reduced to the same thickness as controls (Lewis et al. 1976). Also, in hypothyroid animals granule cells remain in a proliferative phase longer than in controls, resulting in decreased cell differentiation. In contrast, in hyperthyroid animals there is a premature disappearance of the EGL, indicating an early termination in proliferation and early cell differentiation (Nicholson and Altman 1972b).

226. It takes 3 to 4 days in normal euthyroid animals for a migrating granule cell to reach the IGL of the cerebellum (Figure 4-8). In hypothyroid animals the time a migrating granule cell takes to reach the IGL is about half or approximately 2 days. This is most likely due to a 50% decrease in the thickness of the molecular layer (the cell-poor zone between the EGL and IGL) resulting from early differentiation and migration of cells (Rabie et al. 1980).

Figure 4-8 Development of the 3 Layers of the Cerebellum (egl, ml, igl)



On PND 11 (P11), the EGL is still visible. The EGL disappears normally by PND 21 (P21). In contrast, hypothyroid animals still exhibit a visible EGL on P21. ml = mitral layer. Image from Morte et al. (2004)[1]

4.3.8.3 Apoptosis in the Cerebellum

227. Lewis et al. (1976) observed that there was an increase in the number of dying cells in the internal granular layer of 12 day old hypothyroid rats (Lewis et al. 1976). Rabie et al. (1977) also showed an increase in cell death in the IGL of 10, 14, and 21-day old hypothyroid animals. In both normal and treated animals, cell death in the IGL is maximal at 8 days. The greatest difference between normal and hypothyroid animals was observed at 14 days where there was an increase in the pyknotic index by a factor of 20. A reduced ratio of granule cells to Purkinje cells was also observed. A daily dose of 10μg of T_4

[1] Figure Copyright 2004, The Endocrine Society. Used by permission. (Morte et al. 2004)

administered to hypothyroid animals caused the increase in cell death to return to a normal level as well as a correction in the ratio of granule cells to Purkinje cells (Rabie et al. 1977).

228. Using TUNEL staining showed apoptotic activity in the IGL in normal animals from day 2 to 12 with a peak on day 8 and no more apoptotic cells detected on day 22. In hypothyroid animals the peak of apoptosis is also on day 8 but is 4 times higher than controls. Apoptosis in these animals is still detectable on day 22 and does not stop until day 42, indicating not only an increase in the amount of apoptosis but also the duration during development (Xiao and Nikodem 1998b).

229. Although it is clear that thyroid hormone has a dramatic affect on apoptosis during cerebellar development, it is unclear how or why this occurs. There are a number of genes known to be involved in apoptosis, Bcl-2 family proteins being one of the key regulators. Using Western blot it was found that in the cerebellum of hypothyroid animals there is a down- regulation of Bcl-2 and Bcl-x_L, which are both anti-apoptotic proteins. Also, hypothyroidism caused an up-regulation in Bax expression, which is a pro-apoptotic Bcl-2 family protein (Singh et al. 2003a).

4.3.8.4 TH Effects on Purkinje Cell Proliferation and Differentiation

230. Purkinje cells in the cerebellum form a single layer on the margin of the IGL and represent the only cell type that carries information out of the cerebellum. It has been well documented that hypothyroidism during the first postnatal weeks of development causes a reduction in Purkinje cell dendritic arborization and a reduction in synaptogenesis between Purkinje cell dendritic spines and the parallel fibers of granule cells (Legrand 1967; Nicholson and Altman 1972b; Legrand 1982). Legrand also observed that in 14-day old thyroid deficient rats, the inhibition in synaptogenesis was more pronounced at the bottom of the molecular layer, causing a distortion in the normal synaptic organization (Legrand 1967).

231. Recently, work has been done to determine how thyroid hormone regulates Purkinje cell development, specifically looking at TR isoforms. Heuer et al. (2003) found that although both TRα1 and TRβ1 are expressed during the peak of dendrite formation, TRα1 in Purkinje cells is the direct target of T_3 action. Also, T_3 given to cerebellar cultures showed a dose-dependent increase in dendritic outgrowth of Purkinje cells, which was only observed during continuous T_3 exposure. This shows that TH is continuously required and is not simply serving as a molecular switch in the maturation of Purkinje cells (Heuer and Mason 2003). In addition, these histological endpoints could be captured in an integrated EDSP in a manner that could test for the ability to interfere with TRα or TRβ signaling (see discussion below).

4.3.8.5 Methods of Analysis

232. Cerebellar development is well known to be sensitive to thyroid hormone and is the focus of a number of research groups attempting to understand the role of thyroid hormone in brain development (Koibuchi and Chin 2000b). Moreover, because the cerebellum develops largely postnatally in the rat, ongoing developmental screens can easily incorporate measures of cerebellar development to capture endpoints of thyroid hormone action without adding to the number of animals used in the overall battery of tests and screens. Although various aspects of cerebellar development have not been evaluated for their sensitivity to thyroid toxicants, there are a number of easily measured endpoints that may prove useful. Validation of these endpoints for use in a screen for thyroid toxicants would necessarily require evaluating their sensitivity to toxicants that act at different points within the thyroid system. For example, perchlorate or methimazole act almost exclusively on thyroid function, where bisphenol A might act more directly on the TR. Because different TRs mediate different actions of thyroid hormone on different endpoints of cerebellar development, this must be considered when developing a uniform screen.

4.3.8.6 Planimetric Measurements of Cerebellar Development

233. The population of granule cells that ultimately occupy the internal granule layer (IGL) expands in the external granule layer (EGL), then migrate to the IGL (Altman and Bayer 1985). Each of these processes, proliferation and migration, are influenced by thyroid hormone (Koibuchi and Chin 2000b). Potential thyroid toxicants that influence these processes would have effects on brain structure, and therefore would qualify as endpoints reflecting adverse effects of toxicant exposure. Altman and Bayer propose the term "External Germinal Layer" to limit confusion between the two "granule" layers, and to recognize that the EGL is the source of several types of cells that populate the adult cerebellum in addition to granule cells. The sequence of events including proliferation and migration occur in a predictable manner. For example, the planimetric areal measurement of the EGL in postnatal rats increases approximately 10-fold from birth to postnatal day (P) 6 (Altman 1969). Likewise, the width of the molecular layer increases nearly 100-fold during this period (Altman and Winfree 1977). Changes in the planimetric area of the EGL are associated with changes in the depth of this layer. Thus, the EGL is about 5 cells thick at birth, rising to 10 cells thick on P8 to 9 and disappearing by P21 (Altman 1972). Developmental changes in planimetric measurements (or counting numbers of cells in a layer) in response to thyroid disruption could easily be managed within the present EPA guidelines for neurotoxicity testing (EPA 1998).

4.3.8.7 Granule Cell Migration

234. An additional measure of thyroid hormone action in the developing cerebellum is provided by the number of cells found in the molecular layer (between the EGL and IGL) (Manzano et al. 2003a; Morte et al. 2003). This is a simple measurement that can be taken as a surrogate marker for granule cell migration.

4.4 TH-regulated Gene Expression in the Cerebellum

235. There are a number of genes that are known to be regulated by thyroid hormone in the developing cerebellum. Brain-derived neurotropic factor (BDNF) and neurotropin 3 (NT-3) are factors that belong to a group of proteins known to play crucial roles in neuronal differentiation, neurite outgrowth, and synaptogenesis (Lewin and Barde 1996) and have been shown to be affected in hypothyroidism. In hypothyroid rats, BDNF and NT-3 expression is reduced in the cerebellum and replacement of these transcripts prevents the abnormal cerebellar developmental events associated with hypothyroidism (Neveu and Arenas 1996). Although a direct effect of T_3 on the expression of these genes has not been established, there is evidence of TH regulation (Koibuchi et al. 1999a; Koibuchi and Chin 2000a).

236. In a recent paper published by Manzano et al., the effects of GC-1, a thyroid hormone analog that binds selectively to TRβ, on the expression of thyroid hormone target genes in the cerebellum were evaluated (2003). Hypothyroid pups were given either T_3 or GC-1 and the expression of known thyroid hormone responsive genes was analyzed on PND 16. They found that the expression of hairless, Rev-ErbAα, and neurotrophin-3 was significantly decreased and the expression of Reelin was increased, as would be expected. T_3 administration normalized the expression of all the genes whereas GC-1 administration was only able to restore Reelin expression. These results suggest TR isoform-specific regulation of thyroid hormone responsive genes (Manzano et al. 2003a). Specifically, hairless and NT-3 are TH responsive genes regulated by TRα in granular cells, which contain predominantly TRα. Reelin, however, responded to GC-1 in a similar manner as T_3, suggesting regulation through TRβ despite being expressed primarily in granule cells. Reelin has also been shown to be regulated by BDNF, which is also regulated by TH, suggesting that there may be multiple factors involved in Reelin regulation (Koibuchi and Chin 2000a). Rev-ErbAα is expressed in Purkinje cells during the first week of development and plays and important role in development. Deletion of this gene in mice causes a phenotype similar to

hypothyroidism. These results suggest that Rev-ErbAα expression might be specifically regulated by TRα. GC-1 has also been shown to induce Purkinje cell protein-2 (PCP-2) expression, which is another known TH responsive gene expressed in Purkinje cells during development of the cerebellum. It is thought to be regulated by TRβ (Strait et al. 1992; Morte et al. 2002a).

237. As described earlier, the bulk of experiments focused on the role of thyroid hormone in the rodent brain employ models of severe hypothyroidism. This is true for studies focused on the role of thyroid hormone in the control of neural gene expression (Potter et al. 2002; Anderson et al. 2003; Bernal et al. 2003). However, there are a number of strategies for taking the measurements of gene expression and this includes differences in the methods of collection of tissue and methods of quantitation. However, a more difficult issue is to identify thyroid hormone-responsive genes closely linked to adverse effects. Thus, histological endpoints may be preferred initially.

4.5 TH Effects on Cortical Neurogenesis

238. In normal rats, the volume of the cortex and number of glia increases rapidly from PND 5 to PND 20 and remains constant thereafter, whereas neuron number reaches a peak at day 5 and remains constant to day 48. In hypothyroid pups, the mean volume of the cortex, glia number, and neuron number are reduced. T_4 supplementation partially reversed these effects (Behnam-Rassoli et al. 1991).

4.5.1 TH Effects on Cortical Lamination and Barrel Field Differentiation

239. Thyroid hormone causes defects in barrel field cytoarchitecture. Barrel fields are a visibly arranged group of neurons in the somatosensory cortex that are innervated by the animal's whiskers. In hypothyroid animals there is a 27% reduction in total barrel field area compared to control (Berbel et al. 2001). Lavado-Autric et al. also found defects in barrel field organization in pups derived from hypothyroxinemic mothers. They found that in layer IV of the cortex, barrels were not clearly defined and were homogenously distributed (Lavado-Autric et al. 2003).

4.5.2 Methods of Analysis

240. Cerebral cortical neurons are born adjacent to the ventricle (the ventricular zone) then migrate as they differentiate toward the surface of the brain. Early born neurons migrate to positions adjacent to the ventricular zone; late-born neurons migrate farther, past the earlier-born neurons to take positions progressively more superficial. This "upside down" pattern results in the typical laminar appearance of the cerebral cortex. Thyroid hormone insufficiency causes a disturbance in neuronal migration, which is manifested in the adult brain by an apparent disorganization of the cortical lamina. Moreover, if cells are labeled during fetal development using the birth-date marker bromodeoxyuracil (BrdU), it is demonstrable that a significant proportion of cortical neurons migrate to an improper position (Lavado-Autric et al. 2003; Auso et al. 2004a). This observation could be exploited as a screen or test for thyroid disruptions. In a paradigm using fetal and neonatal toxicant exposure, BrdU could be administered to the dam on gestational days 15, 16, and 17. Then, in the context of an EPA guideline study of developmental neurotoxicity, BrdU labeling could be developed to determine whether migration defects occur.

4.6 Thyroid Hormone Action in Oligodendrocytes Differentiation

241. Oligodendrocyte precursors known as oligodendrocytes-type II astrocyte cells (O-2A cells) proliferate and migrate throughout the brain (Raff et al. 1983). Differentiation of these biopotential precursor cells is known to be affected by a number of extracellular factors, including thyroid hormone. Previous work has shown that oligodendrocytes grown in pure cultures without growth factors die after 24 hours. Addition of thyroid hormone to these cultures had no effect on oligodendrocytes survival even with

addition of growth factors (Barres et al. 1994). However, in a recent study done by Jones et al. (Jones et al. 2003), thyroid hormone is shown to be an important survival factor for developing O-2A cells. They prepared primary mixed cell cultures containing not only O2-A cells, but also microglia and astryocytes. They found that without addition of exogenous growth factors these cultures survived for 3 days, indicating that there are survival factors secreted by astrocytes or microglia cells. After 48 hours, T_3 supplementation rescued these developing cells from cell death, indicating that T_3 regulates the expression of unknown factors required for oligodendrocyte differentiation (Jones et al. 2003).

242. In studies done in the rat optic nerve, TRα1 is shown to be important in the normal timing of oligodendrocytes development. In TRα1-/- mice, there are decreased numbers of oligodendrocytes in the P7 and P14 optic nerve and in culture, oligodendrocyte precursors fail to differentiate in response to TH (Billon et al. 2002).

243. The role of thyroid hormone in myelination has been well documented. Hypothyroid rat brains have been shown to have a reduction in total myelin content and a 1 to 2 day delay in myelin protein composition (Walters and Morell 1981). There is also a reduction in the amount of myelin deposited in white matter and fewer myelinated axons compared to controls (Adamo et al. 1990). In hyperthyroid animals, there was a greater accumulation of myelin at 13 days corresponding to an earlier initiation in myelination and a 1 to 2 day acceleration in the myelin protein composition (Walters and Morell 1981).

244. Thyroid hormone is also known to affect a number of genes involved in myelination. 2'3'-cyclic nucleotide 3'phosphodiesterase (CNPase) and myelin-associated/oligodendrocytic basic protein (MOBP) are both genes involved in myelin compaction and have been found to be down-regulated in the cerebellum and corpus callosum of hypothyroid animals (Barradas et al. 2001). Myelin basic protein (MBP) and proteolipidic protein (PLP) are expressed during myelin sheath formation and are also down-regulated in the cerbellum, corpus callosum, striatum and cerebral cortex (Ibarrola and Rodriguez-Pena 1997b; Barradas et al. 2001). Neonatal hyperthyroidism has been found to markedly increase the expression of these myelin genes at 10 days of age, but levels decreased significantly at 17 days. By 70 days of age hyperthyroid animals show decreased myelination, indicating that although myelination is initially increased, it is also terminated earlier (Walters and Morell 1981; Marta et al. 1998).

4.7 Hormone Levels

245. It has been argued, with significant merit, that serum concentrations of thyroid hormones should be an indicator of all thyroid toxicants (DeVito et al. 1999). This endpoint will reveal thyroid toxicants that interfere with thyroid function (by any mechanism), thyroid hormone metabolism (by any mechanism), or TR activation. Chemicals that interfere with thyroid function (e.g., TPO inhibitors) would reduce T_4 synthesis and would suppress serum T_4. Likewise, chemicals that increase thyroid hormone metabolism and clearance from serum (e.g., UDPGT inducers) would cause a reduction in serum T_4 or at least an increase in serum TSH (to maintain normal T_4 levels). Finally, chemicals that interfere with TR activation should alter the negative feedback action of thyroid hormone at the hypothalamus and pituitary, thereby causing a change in serum thyroid hormone levels. Thus, hormone levels are and will remain important indicators of thyroid toxicity. However, thyroid hormone levels change during the early postnatal period and this must be incorporated into screens. For example, T_4 levels in normal rat pups are in the range of 0.5 to 1.0 µg/dL on PND 4 (Goldey et al. 1995; Zoeller et al. 2000), rising to 6 to 8 µg/dL on PND 15, then declining to adult levels of approximately 3 µg/dL. Interestingly, the radioimmunoassay used extensively in toxicological research has a lowest standard of 1 µg/dL. Therefore, measurements in the literature should be carefully evaluated because many of these measurements are below the detectability of the assay kit used.

246. All known thyroid toxicants have been identified by changing serum levels of thyroid hormones (Brucker-Davis 1998). However, changes in serum hormone concentrations do not define the effects that these changes will have on an organism. Thus, while an argument can be made for using serum hormone concentrations as the sole indicator of thyroid toxicity, it will not contribute essential information in the secific effects of the chemical being assessed. Moreover, as was highlighted in chapter 3, many mechanisms of thyroid disruption have not been sufficiently studied and little is know on toxicants that can interfere with TR activation without influencing serum hormone levels. This may be especially true for toxicants that interfere with the TRα isoform, because this isoform, does not contribute significantly to the negative feedback regulation of the pituitary or hypothalamus.

4.8 Additional Histochemical or Biochemical Markers of Thyroid Toxicity in the Developing Brain

247. A large number of endpoints of thyroid hormone action have been described in the developing brain (Bernal 2002; Bernal et al. 2003). These included thyroid hormone-responsive genes, proteins, enzyme activities, and developmental events. It is not likely that these endpoints could be developed for screens that could be incorporated into existing developmental neurotoxicity screens because the tissues have are difficult to prepare (i.e., perfusion with fixative) and the measures of mRNA, protein, or enzyme activity levels may be difficult to standardize.

4.9 Summary of Practical Considerations of Endpoint Capture

248. A number of important practical considerations exist for the assay endpoints discussed above. Perhaps most importantly is interaction of the inherent variability in the assay itself, and the inherent variability in the biology. For example, the RIA for T_4 and T_3 has a very low intra-assay variation, depending on the method used to separate bound from free ^{125}I-T_4. The commercial kits usually have an intra-assay coefficient of variation below 5%. The inter-assay coefficient of variation is usually higher (10-15%); thus, all samples to be evaluated in a single statistical analysis should be evaluated in the same assay, even if multiple standard curves are run to correct for radioactive decay during the counting process itself. In contrast, the biological variation for serum T_4 may be high, especially in development, and may perhaps be affected in a toxicant-specific way. Moreover, because thyroid hormone levels fluctuate throughout the day in rats (Zoeller et al. 1990), it is important that experiments be timed to capture hormone levels at the same time of day.

249. The RIA for TSH is inherently more variable. The intra-assay coefficient of variation of the assay itself usually runs around 10-15%. The inter-assay coefficient of variation is greater. Moreover, TSH is released in a pulsatile manner and fluctuates widely in serum over minutes and hours (Mantzoros et al. 2001). Thus, serum TSH measurements will inherently be more variable than serum T_4 and the statistical power will be lower given the same number of animals. We simply do not know the inherent variability in the endpoints of thyroid hormone action discussed above. However, if some of these endpoints were to be evaluated for their suitability/reliability in toxicity screens/tests, this practical aspect would clearly have to be carefully evaluated. It is important to recognize also that immunoassayable TSH does not necessarily indicate bioactive TSH (Persani et al. 1998). Different degrees of sialylation of the carbohydrate moieties of TSH account for differences in biological activities. Perhaps because of this, as well as differences in the primary sequence of TSH itself, heterologous radioimmunoassays (i.e., assays in one species using an antibody against TSH from a different species) should be very carefully validated prior to use.

4.10 *In vitro* Screens

250. There are no cell lines or primary cells that have been validated for use as a thyroid screen in a manner similar or analogous to the E-Screen for estrogen activity, though there are possibilities. In part, this may be because the general focus has been on the ability of chemicals to affect thyroid function, not thyroid hormone action. However, as more chemicals are found to influence TR function, it may be necessary to develop *in vitro* screens (IPCS 1990). The following represent potential *in vitro* assays for thyroid toxicants.

4.10.1 Primary Cultures

251. A number of primary cultures have been employed to study the role of thyroid hormone in brain development and in the development or physiology of various tissues. An important source of primary cells is fetal cortical neurons. For example, these cells (harvested on gestational day 16) were shown by McKay and his colleagues to retain the capacity to differentiate into neuronal or glial lineages (Johe et al. 1996). Moreover, they found that thyroid hormone could increase the formation of glial cells at the expense of forming neurons. Another example is provided by Denver et al. (1999), who showed, using primary cortical cells harvested on gestational day 16, that the transcription factor BTEB is thyroid hormone responsive and may be involved in the regulation of neurite outgrowth.

252. Cerebellar granule cells are also an important source of primary cultures. One of many examples is work from the lab of Thompson (Potter et al. 2001) who studied the regulation of synaptotagmin-1 in primary cerebellar granule cells. Likewise, primary cultures of astrocytes harvested from the early postnatal cerebellum have been used quite extensively. These cells have provided the basis of the work by Farwell and Leonard on thyroid hormone actions on actin polymerization and vesicular recycling (Leonard et al. 1994; Leonard and Farwell 1997; Farwell and Dubord-Tomasetti 1999b, 1999a; Stachelek et al. 2000; Stachelek et al. 2001). Cardiac myocytes (Bahouth 1991; Dillmann 2002; Neves et al. 2002) and lung tissue (Mendelson and Boggaram 1991) also have proved to be important primary cultures to study thyroid hormone action.

4.10.2 Cell Lines

253. A very large number of cell lines have been employed to study thyroid hormone action. It is neither practical nor informative to review all of these. Therefore, we will review some of the strategies for using different cell lines.

254. Studies of TR mechanisms require cells without endogenous TRs and which are relatively easy to transfect with one or more constructs. One cell line often used in this research is made up of 293T cells (Shibusawa et al. 2003b; Shibusawa et al. 2003a). These are human cells that have been stably transfected with the simian virus T-antigen, allowing it to proliferate rapidly. Moreover, it carries selectable marker genes to increase its utility under conditions of transient transfection studies. In contrast, N-tera-2 (NT-2) cells are derived from a human testicular carcinoma, but possess neuronal precursor characteristics and can be used to study fate specification and the role of thyroid hormone in differentiative events. Recently, Chan (Chan et al. 2003) has characterized the expression pattern of the TRs both before and after terminal differentiation. In this regard, these cells are similar to PC12 cells, which have also been used to study thyroid hormone action (Munoz et al. 1993).

4.10.3 In vitro Binding Assays

255. *In vitro* binding assays can be used as potential screens for chemicals that bind to TRs. The classical binding assays have used nuclear extracts from a variety of tissues and cell lines expressing TRs (e.g., (Gauger et al. 2004a). More recent studies have used various TR isoforms expressed in *E. coli* or

translated *in vitro* (Cheek et al. 1999). These assays require separating bound from free hormones using either filtering or chromatographic methods. Either separation method is cumbersome and time-consuming. More recent advances have used solid-state binding assays using specific isoforms of TRs. The solid-state binding assays developed allow for high throughput screening. In the solid-state binding assays, the TR is coupled to either a multiwell plate or to beads. Coupling of the receptors to plates or beads readily enables the separation of free and bound ligands without the use of either filtering or chromatographic methods. Only three of the four TR isoforms have ligand-binding capability and two of these (TRβ1 and TRβ2) have identical ligand-binding domains. Binding assays are expected to have a low rate of false positives. False negatives can occur if the chemical requires metabolic activation, if solubility problems are encountered, or if the chemical affects TR function without binding to the ligand binding domain of the receptor.

256. Thyroid hormone receptors are structurally conserved among the vertebrates. However, their interactions with ligands, DNA, and accessory proteins are quite complex and it is not likely that TRs from all vertebrates will respond identically. However, considering the paucity of pharmaceuticals directed at TRs, there are very few studies upon which to make predictions concerning the uniformity of toxicant effects on TR behaviors.

4.10.4 Transfection and Transformation Assays

257. One of the problems with TR binding assays is that they cannot differentiate between agonists and antagonists. Alternative assays that would examine effects on receptor function and differentiate between agonists and antagonists are systems in which a specific TR is transfected into a mammalian cell line along with a reporter gene, typically coding for luciferase, beta-galactosidase, or choline acetyl transferase (Zhang and Lazar 2000). Transformed yeast cell lines containing TR gene constructs have also been developed. In these systems, T_3 or other TR ligands bind and activate the receptor, which then interacts with specific response elements upstream from the reporter gene and enhances its transcription. The increased transcription is determined by increased enzymatic activity of the reporter gene product, e.g., luciferase. Chemicals can be tested alone or in combination with T_3 to determine agonist or antagonist properties. Similar systems have been used to examine the interactions of TR with different response elements (Mangelsdorf and Evans 1995), different cofactors (Yen 2001), and with phosphorylation of TR (Bassett et al. 2003; Mendez-Pertuz et al. 2003; Stevens et al. 2003). Although these systems have not been used for screening for environmental chemicals that are TR ligands, similar screens have been developed for estrogens and androgen agonists and antagonists.

258. There is some evidence that thyroid hormone receptors act predominately as heterodimers with RXR (Mangelsdorf and Evans 1995); however, this may be promoter-specific (Koenig 1998; Wu et al. 2001b). Hence, chemicals might alter TR activation by altering RXR pathways. TR activation is also regulated by phosphorylation (Yen 2001); DNA binding may be dependent upon TR phosphorylation. In designing a screen for TR ligands, chemicals may have different effects depending on the TR transfected, the response element used, and their interactions with potential heterodimers. Because of the complexity of this system, several screens would have to be incorporated to account for the multiplicity of interactions of the different TR isoforms. An advantage of the transfection assays is that chemicals that alter TR activation through mechanisms not involving direct binding to TR would be detected in these assays. Another advantage of these assays is that they are readily adapted to high throughput screens.

259. A significant disadvantage of these *in vitro* screens is the potential lack of metabolic capability of the cells used in the assays. It is possible that the metabolites of some chemicals, and not the parent compound, would produce these effects. For example, parent BPA appears to be a TR antagonist (Moriyama et al. 2002), but the polyhalogenated derivatives are agonists (Kitamura et al. 2002). The cell lines typically used in these assays have limited ability to metabolize the test compounds, particularly persistent organic pollutants such as the polychlorinated biphenyls (PCBs) and the dioxins. The

transformation assays in yeast have additional drawbacks in that for many chemicals, entry into the yeast is limited because of the cell wall.

4.10.5 GH_3 Cell Assay for Thyroid Hormone Action

260. An *in vitro* bioassay has been designed that can detect compounds that interfere with TR signaling much the way the MCF-7 cells are used in the E-SCREEN (Hohenwarter et al. 1996). GH_3 cells have TRα1, TRβ1, and TRα2. This assay uses the rat pituitary somatotroph cell line GH_3. Proliferation of these cells is dependent on thyroid hormone when plated at low-density in serum-free medium (Hohenwarter et al. 1996). One form of the assay measures cell proliferation in response to TR agonists by the determination of the transformation of monotetrazolium (MTT) tetraxolium salt into MTT fromazan by mitochondrial enzymes. This assay is performed on microwell plates and can be considered a high throughput screen. Although this assay is relatively new, it has the potential to provide information as a screen for chemicals that activate TR. In the presence of thyroid hormone, this assay can detect TR antagonists.

4.10.6 FRTL-5 Cells

261. These cells were derived from Fisher rat thyroid tissue and have been maintained in culture. These cells can be used to test the ability of toxicants to affect several aspects of thyroid physiology. However, although these cells can concentrate iodide, they cannot organify it (perhaps because there is no colloid). Thus, these cells could be used as *in vitro* methods of identifying iodide uptake inhibitors or potentially those chemicals that inhibit other thyroid functions (e.g., Tg synthesis, cAMP production in response to TSH).

4.11 Effect of Xenobiotics

262. During the past 3 years, several research papers have appeared reporting on the ability of various xenobiotics to influence thyroid hormone-regulated transcription. These xenobiotics include various PCBs or mixtures of PCBs (Iwasaki et al. 2002; Bogazzi et al. 2003; Yamada-Okabe et al. 2003), bisphenol A (Kitamura et al. 2002; Moriyama et al. 2002), and dioxin (Yamada-Okabe et al. 2003). The mechanism(s) by which these xenobiotics affect TR-mediated gene regulation are diverse, indicating that screens for TR-active compounds must be broad in nature. For example, parent bisphenol A and its polyhalogenated derivatives bind to the TR and exert either an agonist or antagonist effect. In contrast, 2,3,7,8-tetrachloro-p-dioxin (TCDD) augments T_3 action on the TR (Yamada-Okabe et al. 2004) but there is little evidence that TCDD binds to the TR. Likewise, specific hydroxylated PCBs can either suppress (Iwasaki et al. 2002; Miyazaki et al. 2004) or augment (Bogazzi et al. 2003) TR activation, also without binding to the TR and displacing T_3 (Gauger et al. 2004a). Interestingly, Miyazaki et al. (Miyazaki et al. 2004) have proposed the novel hypothesis that PCBs can cause the TR to dissociate from DNA. This intriguing hypothesis has two important implications. First, it implies that PCBs (and perhaps other polyhalogenated aromatic hydrocarbons) bind to an allosteric site on the TR, which may in fact be a site targeted by endogenous factors. Second, it implies that PCBs cause derepression of TR-regulated genes without activation. Thus, depending on how the experiment is designed, one may conclude that PCBs inhibit thyroid hormone action or exert thyroid hormone-like effects.

263. These findings demonstrate the importance of incorporating screens that measure the ability of xenobiotics to interfere with thyroid hormone action on the receptor. An argument against incorporating transcription assay(s) into a screen for potential thyroid toxicants is that these chemicals would also affect circulating levels of thyroid hormones. For example, PCBs and dioxins are well known to influence thyroid hormone levels (Zoeller 2001). However, changes in circulating levels of thyroid hormone are not, in and of themselves, considered to be adverse effects (Strawson et al. 2004). Moreover, BPA appears to

be a potentially important thyroid toxicant in terms of its mechanism of action and its presence in the environment, yet previous studies have not found that BPA can alter circulating levels of thyroid hormone (Takagi et al. 2002; Tyl et al. 2002).

4.12 Advantages and Disadvantages of *In Vivo* Screens/Tests

264. The advantages of using live intact rodents in screens and tests include:

- Mammalian ADME (absorption, distribution, metabolism, and elimination) is present so any effects observed are realistically predictive (versus worst case theoretical outcomes)
- There is a lower risk of false positives (the target site in an intact animal may never see the active moiety) and of false negatives (if the metabolite is the proximate toxicant) since the metabolic machinery is present to generate the metabolite(s) in intact animals
- The target(s) of the test material, such as hypothalamus, pituitary, thyroid, liver, kidneys, etc. are present and functional, so the screen or test is not limited to assessing a single or only a few mechanisms of action and/or target sites
- Effects in intact rodent models can be more confidently extrapolated to humans; the intact model is much more predictive of human risk
- Subtle effects or effects in other systems such as alterations in behavior, changes in immune response, developmental effects, reproductive effects, can be detected
- Effects on circulating levels of hormones of interest can be determined
- Results from *in vivo* screens can be used for a decision point to proceed (or not) to Tier II tests; *in vitro* screens cannot be used for such a decision point (EDSTAC 1998).

265. The disadvantages of using live intact rodents (versus using an *in vitro* system) include:

- Cost and time (greater costs and longer times for *in vivo* studies)
- Determination of mechanism (or even mode) of action is difficult to impossible in an *in vivo* study
- Determination of primary site of effect and/or identification of causal effects versus downstream subsequent resulting effects is difficult to impossible
- There is no way to evaluate large numbers of chemicals except by repeated or longer studies (vs. high throughput *in vitro* assays).

4.13 Extrapolating Results of Thyroid Toxicity Studies in Rodents to Human Health.

266. Enough is known about the thyroid system among all vertebrate taxa that very clear generalizations can be made concerning the similarities and differences across vertebrates, as well as to make recommendations for areas of research that would improve the degree to which research performed in one species can be generalized to all vertebrates including humans. The following discussion is but a brief and superficial treatment of this topic. It applies to extrapolations across vertebrates in general, not solely between rodents and humans.

267. The degree to which an individual toxicant will affect thyroid hormone in different vertebrate species will depend on a number of known variables including toxicant exposure, metabolism, and mode of action. Toxicant metabolism may differ among different species to a large degree. In addition, for any one mode of action, the relationship between the dose-response of the chemical on the key events may differ considerably among vertebrates. For example, Doerge and Sheehan (2002) showed that thyroperoxidase could be inhibited by nearly 80% without affecting serum T_4. This type of information is not available across the vertebrates, so it is possible that this value is not valid for all animals (including humans). Likewise, the degree to which phase II enzymes in the liver must be induced before causing an effect on circulating levels of T_4 may well be species-specific. Finally, differences among vertebrates in the serum half-life for T_4 and T_3, the basal production of the hormone, and the ability of various compensatory mechanisms to respond to toxicant effects, all contribute to potential dose-response differences for any single toxicant on the thyroid system across the vertebrates.

268. However, since HPT axis function is relatively conserved across vertebrate taxa, observed effects in one species will be considered with the assumption that the same toxicant may affect other vertebrate taxa. Extrapolation of potency across species remains problematic due to a lack of comparative studies as described above. A systematic evaluation of chemicals, which have both rodent and human data, should be undertaken to determine whether the data are available to make clear and justifiable decisions about the extrapolation of rodent data to humans in specific cases. Furthermore, a similar comparison across vertebrate taxa should be done and the data needed to design such a comparison should be considered. Issues surrounding extrapolation across vertebrate taxa are discussed in the following paragraphs.

269. In general, thyroid toxicants can reduce circulating levels of thyroid hormones by any one of a number of modes of actions. These include acting directly on the thyroid gland to inhibit iodide uptake at the sodium iodide symporter (NIS), inhibiting TPO, and inhibiting cathepsins that liberate T_4/T_3 from Tg. In addition, toxicants can enhance the expression of metabolizing enzymes (UDPGTs, Sulfotransferases) in liver. Toxicants are known to displace T_4 from serum binding proteins as well. Finally, toxicants are known to influence deiodinase activity (e.g., D1), but this may not always be a direct action.

270. It is clear from human studies that small differences in the DNA sequence of structural or regulatory regions of genes coding for proteins involved in thyroid function or thyroid hormone signalling, can be associated with differences in the way the HPT axis functions. Therefore, it is likely – though not formally tested – that any single toxicant (e.g., perchlorate that interacts with the NIS) may well exert different potencies of effects in different individuals based on genetic variation in specific genes (Scinicariello et al. 2005). By inference then, it is also clear that the potencies of specific toxicants that interact with key proteins in thyroid function or thyroid hormone signalling may be different among vertebrate species within a class, or between taxa. This particular issue remains very empirical. For example, the fact that the NIS in humans shares a certain degree of identity with NIS in fish would serve as the basis to predict that it will be a target of perchlorate inhibition. However, a potency series would have to be conducted to empirically determine the relative potency of various anions on the fish NIS. Thus, the ability to extrapolate to human health, or to generalize data obtained from work on a specific taxa to other taxa, will depend on a number of factors that include a thorough analysis of the genetic variation in the key proteins that represent targets of toxicant actions, metabolic and life stage differences, as well as empirical data characterizing the ability of a toxicant to interact with the homologous protein across vertebrates. The limited number of thyroid toxicants studied across multiple taxa, indicate that the effects of toxicants across taxa may be seen with respect to the thyroid system, but they may be different.

271. A much more complex issue relates to the effects of toxicants on the pattern of changes in thyroid hormones and thyroid histology induced by a particular toxicant across species. Specifically, some toxicants may induce different patterns of responses across various taxa, including between rodents and humans. This possibility is supported by the observation that different taxa appear to rely to different

degrees on various mechanisms within the HPT axis. For example, rodents have less stored thyroid hormone in the colloid compared to adult humans; therefore, it is predictable that propylthiouracil (PTU) treatment would require a longer duration of exposure in humans (at least in adults) than in rodents to exert an effect on serum hormone levels. Likewise, the ancillary reports presented in Appendix A (Zoeller 2005) and Appendix B (Feder and Feng 2005) of this DRP clearly demonstrate that different toxicants exert a different profile of changes in serum hormone levels in rodents. This observation is almost certainly dependent on the mechanism of action of specific thyroid toxicants. As a case in point, Glatt et al. (2005) showed that three thyroid toxicants exert different effects on the expression of the sodium-iodide symporter (NIS). Specifically, excess iodide and Phenobarbital (PB) both caused a significant decrease in NIS expression in the thyroid gland. In contrast, PTU caused an increase in NIS expression in the thyroid gland. However, both PTU and PB caused a decrease in circulating levels of T_4, while excess iodide did not. We do not fully understand why different toxicants exert different effects on the hormonal profile of the HPT axis of a single experimental animal (e.g., rodents). It is unlikely that we will understand fully enough to make predictions whether different vertebrates will respond to the same chemical in the same manner until more data are obtained and compared across chemicals, their modes of actions, and the pattern of effects across different vertebrate taxa. However, because this will enhance our ability to interpret the results of experimental studies to human health, this will be an important area for investigation as more data are generated in the scientific community and through the currently proposed assays for thyroid that are being validated.

4.14 Balancing the Timing and Duration of Exposure with the Timing of Endpoint Acquisition

272. The literature reviewed in this DRP clearly demonstrates that, in mammals and in other vertebrates, thyroid hormone insufficiency produces different effects at different times during development. This implies that exposure to thyroid toxicants disrupting thyroid hormone action would exert different effects at different times during development. Considering this, it is extremely important to take into account the timing of exposure and endpoint capture when adding thyroid toxicity endpoints to existing mammalian screens and tests (e.g., OECD Test Guidelines). For example, endpoints of neurodevelopment could be added on to one- and two-generation tests or the TG 426 Developmental Neurotoxicity Study. The key issue is that timing of toxicant exposure be designed to capture endpoints that are relevant for the timing of exposure.

273. A second important consideration is that of the ability of the HPT axis to compensate for perturbations in the system. For example, a toxicant that induces liver UDPGTs may cause an immediate decrease in circulating levels of thyroid hormone, but over time, these may be compensated by changes in TSH and by changes in the thyroid gland (increased NIS and increased efficiency of TSH signalling) that would restore circulating levels of thyroid hormone to control levels. However, it is important to recognize that this would not necessarily mean that thyroid hormone action had not been disrupted, producing adverse effects. However, the transient decrease in circulating levels of thyroid hormone during sensitive lifestages, especially if it occurred during development, may well have impacts on development.

4.15 A Consideration of Dose-Response Relationships Between Toxicants and Thyroid Endpoints

274. Given the complex nature of the thyroid system, it is useful to consider existing dose-response relationships and what might underlie the different dose-response shapes. This is important because non-linear and non monotonic dose responses are common both in the endocrine system at large, and in the thyroid system specifically. For example, the thyroperoxidase inhibitor PTU produces a nearly linear dose effect on serum T_4, and serum TSH responds logarithmically to this T_4 decline (Zoeller 2005). Moreover, changes in thyroid weight and histology parallel hormone changes. Far less information is exigent concerning the dose effect of PTU on endpoints of TH action. T_3 exerts a linear dose effect on gene

expression in transient transfection systems, suggesting that the linear dose effect of PTU on serum T_4 might also be associated with a linear dose effect of PTU on endpoints of TH action. However, this has not been studied, and one can imagine that compensatory responses in serum binding proteins, transporters, receptors and co-factors may considerably alter the shape of the dose-response curve of PTU on thyroid hormone response genes in tissues.

275. In contrast, consider the apparent dose effect of perchlorate, an inhibitor of the sodium-iodide symporter, on serum thyroid hormone (Zoeller 2005). Low doses of perchlorate appear to cause an increase in serum T_4, which is both paradoxical and currently unexplained. This observation has been reported for rodents (Thuett et al. 2002) and in humans (Crump et al. 2000; Braverman et al. 2005). However, clearly, higher doses of perchlorate cause a reduction in thyroid hormone levels.

276. Polychlorinated biphenyls (PCBs) produce a nearly linear dose effect on serum T_4 levels, but TSH does not respond to these changes (Zoeller 2005). Moreover, thyroid histology does not respond. Finally, endpoints of thyroid hormone action following PCB treatment are quite enigmatic in that some measures (e.g., RC3/Neurogranin) appear to reflect an agonistic effect of PCB exposure, despite a reduction in serum T_4 (Zoeller et al. 2000), but others (e.g., hearing) appear to reflect the PCB-induced T_4 deficit (Goldey et al. 1995; Crofton 2004).

277. Finally, consider the recently reported dose effect of bisphenol A (BPA) on serum T_4 (Zoeller et al. 2005). In this case, BPA produces a very flat increase in serum T_4 without a concomitant suppression of TSH. Moreover, an endpoint of thyroid hormone action (RC3/Neurogranin expression) is increased in a manner consistent with the increase in serum T_4. Considering that BPA is shown to be a thyroid hormone receptor antagonist *in vitro* (Moriyama et al. 2002), one interpretation is that BPA causes serum T_4 to become elevated by partially inhibiting the negative feedback effect of thyroid hormone on the pituitary (and hypothalamic) thyroid hormone receptor (TR). Moreover, the flat dose response of BPA on serum T_4 levels may be attributable to the mechanism by which BPA antagonizes T_3-induced TR activation. Specifically, BPA causes the TR to recruit a corepressor (N-CoR), which is likely to be in limited abundance. Thus, the antagonism will saturate in direct proportion to N-CoR abundance. This type of dose response may be characteristic of this type of antagonism. For example, RU 486, an indirect antagonist of the progestin receptor, exerts its antiprogestin activity (Liu et al. 2002; Schulz et al. 2002) in the same way that BPA affects the TR. Like the effect of BPA on serum T_4, RU 486 produces a flat dose response on progestin receptor-regulated gene expression (Liu et al. 2002; Schulz et al. 2002). Thus, the shape of this dose response (i.e., flat) does not abrogate its therapeutic efficacy. Likewise, the flat dose response of BPA on serum T_4 may be no less important.

278. This brief discussion emphasizes the need to consider the mechanism of toxicant action on the HPT axis when interpreting the dose-response relationship between toxicant and measures of thyroid function or thyroid hormone action. While our concepts of the function of the HPT axis are based on experiments using the TPO inhibitors PTU and MMI, the same relationship does not hold for toxicants that act on the HPT axis at different sites.

4.16 Conclusions and Recommendations

279. Mammalian thyroid hormone function and homeostasis is controlled by a complex and interactive system that encompasses hormone synthesis, release, transport, local metabolism, and catabolism. To date, the evaluation of adverse effects of environmental chemicals on the thyroid have focused on parameters of overall function, since the mechanism of action of a thyroid disruptor may involve any of these processes. As a result, the identification of a simple thyroid disrupter screening assay, either *in vivo*, *ex vivo,* or *in vitro,* is not straightforward. A summary of candidate rodent thyroid assays, including major endpoints, target effects, advantages, and disadvantages, is presented in Table 4-3 below.

280. Table 4-4 below shows points of thyroid disruption in mammals. Specific points of thyroid disruption are listed in the left column, coupled to listings of endpoints by which they are characterized, the ultimate effect of disruption by each mechanism, whether assays are available to detect each point of disruption, and the status of this assay.

Table 4-3 Existing or Potential *In vivo* and *In vitro* Assays - Mammals

Assay Name	Species	Major Thyroid-Related Endpoints	Target Effects Relevant to the Thyroid System	Status of the Assay	
				Advantages	Disadvantages
***In Vivo* Assays**					
OECD TG 407 Repeated dose 28-Day Oral Toxicity Study OECD TG 414 Prenatal Development Toxicity Study OECD 415/416 1- and 2-Gen Reproductive Toxicity Studies OECD 421/422 Reproductive/ Developmental Toxicity Study OECD 426 Developmental Neurotoxicity Study	Rat The specific treatment paradigms are described in Section 4.3 for each of these rodent assays.	T_4, T_3, TSH, thyroid weight and histology, testes histology, sperm count, occurrence of tumors in chronic toxicity studies. Timing of endpoint acquisition is gauged to obtain measures of thyroid disruption at optimal times (see text).	Changes in circulating levels of TH, hypertrophy or proliferation of thyroid follicules, development of testes	Straightforward add-on; circulating levels of TH can be related to human thyroid function; follicular proliferation reflects TSH increase; thyroid histology not particularly sensitive to confounders , tumor occurrence important cancer endpoint.	Time course data lacking for compensatory changes; response to stress not characterized

Assay Name	Species	Major Thyroid-Related Endpoints	Target Effects Relevant to the Thyroid System	Status of the Assay	
				Advantages	Disadvantages
***In Vivo* Endpoints as Possible Add-Ons**					
Perchlorate discharge	Rat	Iodine uptake in the thyroid	Tyrosine iodination; circulating T_4 levels	Well-characterized in both animals and humans; low false positive. Very specific for compounds that interfere with iodine uptake into the thyroid gland.	Requires use of radioactivity in animals. Is a single endpoint for an *in vivo* assay.
TRH challenge	Rat	HPT axis integrity	Feedback mechanism; circulating TH levels	Well-characterized in both animals and humans; can distinguish between pituitary and hypothalamic effects. Very specific for compounds that interfere with the pituitary response to TRH.	Cannot easily be combined with additional endpoints.
Developing brain morphology and biochemistry	Rat	Decreased maternal, fetal, and neonatal T_4; decreased perinatal brain weight; morphological abnormalities in cells; changes in neurotransmitter levels	Rodent brain development	Large number of potential anatomical endpoints available for validation, including neuronal migration, proliferation, etc. These are covered in the text.	Labor intensive; some specific endpoints are dependent on both fetal/neonatal T_4 levels

Assay Name	Species	Major Thyroid-Related Endpoints	Target Effects Relevant to the Thyroid System	Status of the Assay	
				Advantages	Disadvantages
Myelination	Rat	The developmental timing of myelin acquisition. Ratio of oligodendrocytes to astrocytes in white matter tracts.	Brain Development	There are simple histochemical stains that can provide a measure of myelination. The ratio of oligodendrocytes to astrocytes is quite sensitive to TH insufficiency.	Is not a validated measure of TH disruption.
Behavioral testing	Rat	Acquisition of developmental landmarks, body weight; auditory function; motor activity	Mammalian brain development	Detects integrated developmental changes in CNS	Labor-intensive; specialized equipment; may not be specific; high false negative; only positive when significant decreases in T_4 levels occur
Male reproductive system development	Rat	Testis weight; sperm count	Development of the male reproductive system	Easy to measure; add-on assay	Not specific for thyroid toxicants; longer study duration; observed only when there is a significant decrease in TH concentration
TH release	Thyroid gland hormone content; circulating hormone levels (total and free T_4 and T_3, TSH); thyroid histopathology	Disturbs thyroid status, including circulating hormones, which result in reflexive response of the HPT axis.	Yes; but there are no toxicants that directly affect TH release	The endpoints of interest would likely be indicative of other modes of action	TH release

Assay Name	Species	Major Thyroid-Related Endpoints	Target Effects Relevant to the Thyroid System	Status of the Assay	
				Advantages	Disadvantages
Glucuronidation - Clearance	Liver enzyme (UDPGTs) induction; altered serum hormone half-life; serum total and free T_4, T_3, and TSH, thyroid histopathology; thyroid gland hormone content	Disturbs serum hormone concentrations; causes a reduction in thyroidal content of T_4 and T_3, causes reflexive changes in serum TSH	Yes; measures of hormone half-life not often performed	Well developed assays.	Glucuronidation - Clearance
Tissue uptake	Hormone content in tissues; transfer of labeled hormone into tissues	Would alter the thyroid hormone status of individual tissues, perhaps selectively.	No	Selective T_3 and T_4 transporters are identified; little is known about their actions	Tissue uptake
Deiodinase	Measurement of types I, II, and III deiodinase in tissues	D1 and D2 are outer-ring deiodinases controlling tissue levels of T_3. D3 is an inner ring deiodinase controlling conversion of T_4 to reverse T_3 and T_3 to T_2.	Yes	Assays of deiodinase activity in tissue or cells is routine; isolated enzymes are complicated	Deiodinase

Assay Name	Species	Major Thyroid-Related Endpoints	Target Effects Relevant to the Thyroid System	Status of the Assay	
				Advantages	Disadvantages
	Serum T_3; tissue levels of T_4 and T_3	Serum T_3 is largely produced by peripheral conversion of T_4 to T_3 by D1. Therefore, serum T_3 is more a marker of D1 activity than it is of thyroid function.	Yes	Serum T_3 is routine; tissue levels of T_4 and T_3 are not routine assays and require optimization and calibration.	Deiodinase
***In Vitro* Assays**					
Serum protein binding (TTR)	Rat	Displacement of T_4 from TTR	Transport of TH to peripheral tissues; development of the brain; transport to fetus	Well-characterized; can be modified for high throughput; predictive of chemicals that may alter fetal concentrations of T_4	Serum protein binding (TTR)
In vitro receptor binding	Could use nuclear extracts, recombinant protein, or ligand binding protein.	Binding of T_3 to the nuclear receptor	Thyroid Hormone action.	Solid state binding assays available; low rate of false positive; appropriate for high throughput	Receptor binding not fully characterized as a mechanism of toxicity; only chemicals that interfere with binding will show as positive; no metabolic activation; solubility

Assay Name	Species	Major Thyroid-Related Endpoints	Target Effects Relevant to the Thyroid System	Status of the Assay	
				Advantages	Disadvantages
Expression Assays	Yeast; mammalian or other vertebrate cells	Receptor binding of T_3	Thyroid Hormone Action	Can determine agonist or antagonist properties; system can be manipulated, optimized, etc.; readily adapted to high throughput	limited metabolic activity; cell wall (yeast)
GH_3 cell assay	Rat	Growth/normal morphology of cell signals agonist activity	Thyroid Hormone action	High throughput adaptability; uses fewer animals; can detect agonist or antagonist activity	Specific for TR binding; chemicals that interfere with thyroid function will not show positive.
Serum Binding Proteins	Protein source could be recombinant or ligand binding domain.	T_4 binding to serum proteins	Circulating levels of TH	Does not use animals. High throughput. Some authors suggest that TTR/TBG binding predicts binding to other T_3/T_4 binding proteins.	Many thyroid toxicants do not bind to serum binding proteins. TTR binding remains to be validated as a predictor of toxicity.
Thyroperoxidase inhibition	Protein source can be recombinant or on histological sections of thyroid tissue.	Thyroid hormone synthesis.	Circulating levels of TH.	May be adaptable to high throughput. Large numbers of known toxicants act by this mechanism. Likely low numbers of false positives.	Chemicals that do not act by this mechanism would not be detected.

Table 4-4 Points of Thyroid Disruption in Mammals

Site of Disruption	Endpoints of Interest	Target Effect of Disruption	Assays Available	Assay Status
Na/Iodide Uptake	Radioactive iodide uptake; Circulating hormone levels (total and free T_4, T_3, TSH); Thyroid gland hormone content; Thyroid gland histopathology	Disturbs thyroid status, including circulating hormones, which result in reflexive response of the HPT axis.	Yes; routine except iodide uptake inhibition	Assays for endpoints of interest are routine. Relation between I-uptake and TH synthesis not known
TPO inhibition	Perchlorate discharge test; TPO inhibition (*in vitro)*; thyroid gland hormone content; circulating hormone levels (total and free T_4 and T_3, TSH); thyroid histopathology	Disturbs thyroid status, including circulating hormones, which result in reflexive response of the HPT axis.	Yes; routine except iodide uptake inhibition	Assays for endpoints of interest are routine. Relation between TPO inhibition and TH synthesis not known
TH release	thyroid gland hormone content; circulating hormone levels (total and free T_4 and T_3, TSH); thyroid histopathology	Disturbs thyroid status, including circulating hormones, which result in reflexive response of the HPT axis.	Yes; but there are no toxicants that directly affect TH release	The endpoints of interest would likely be indicative of other modes of action
Serum Binding Proteins	T_4 displacement from serum binding proteins (TTR, TBG)	Causes a reduction in circulating levels of total T_4, but often does not cause a reduction in serum free T_4.	Yes; but relationship to tissue levels of thyroid hormone is poorly understood	Well developed binding assay for these proteins. May be a screen for T_4-like toxicants.
Glucuronidation - Clearance	Liver enzyme (UDPGTs) induction; altered serum hormone half-life; serum total and free T_4, T_3, and TSH, thyroid histopathology; thyroid gland hormone content	Disturbs serum hormone concentrations; causes a reduction in thyroidal content of T_4 and T_3, causes reflexive changes in serum TSH	Yes; measures of hormone half-life not often performed	Well developed assays.

Site of Disruption	Endpoints of Interest	Target Effect of Disruption	Assays Available	Assay Status
Tissue uptake	Hormone content in tissues; transfer of labeled hormone into tissues	Would alter the thyroid hormone status of individual tissues, perhaps selectively.	No	Selective T_3 and T_4 transporters are identified; little is known about their actions
Deiodinase	Measurement of types I, II, and III deiodinase	D1 and D2 are outer-ring deiodinases controlling tissue levels of T_3. D3 is an inner ring deiodinase controlling conversion of T_4 to reverse T_3 and T_3 to T_2.	Yes	Assays of deiodinase activity in tissue or cells is routine; isolated enzymes are complicated
Deiodinase	Serum T_3; tissue levels of T_4 and T_3	Serum T_3 is largely produced by peripheral conversion of T_4 to T_3 by D1. Therefore, serum T_3 is more a marker of D1 activity than it is of thyroid function.	Yes	Serum T_3 is routine; tissue levels of T_4 and T_3 are not routine assays and require optimization and calibration.
Thyroid hormone receptor binding	T_3 displacement	New studies are showing that chemicals are able to bind to the TR with environmentally relevant affinities	Yes	Assays are well characterized, two types of receptors with several isoforms each
Thyroid hormone receptor activation/repression	TH-responsive gene regulation	New studies are showing that chemicals that cannot bind to the TR still alter TH-regulated gene expression	Yes	Assays are well developed. Choice of TRE may not be trivial. Not routinely used for thyroid toxicants.

281. Based on this information, it appears that the determination of serum free and total T_4, T_3, and TSH, in combination with thyroid weight and histology, comprises the most informative, if not complete, approach to an initial determination of *thyroid function*. However, in order to optimize the information obtained from these assays, a more complete characterization of the sampling time course and appropriate dosing regimen is important. In addition, information concerning the effect of handling stress, postnatal age, and estrous cycle on serum concentrations of thyroid hormones will aid in identifying weak agonists and antagonists. As a simple *in vivo* screen, the current design of the Female or Male pubertal assay, the OECD TG 407, or the 15-Day Adult Male Screen, as described in Section 4.3, is satisfactory. Examination of circulating TH (total and free T_4, T_3) can identify effects on synthesis, transport, and/or elimination, with evaluation of TSH, thyroid weight, and thyroid histology providing additional evidence of altered synthesis and release. The addition of hepatic microsomal glucuronidase activity to this assay would add a specific indicator for TH catabolism, which may aid in providing a more complete initial characterization of thyroid disrupting activity.

282. The major drawbacks to using an *in vitro* mammalian thyroid screen are the extreme specificity of mechanistic endpoint, the absence of metabolic activation, and solubility issues. Although *in vitro* thyroid assays are unlikely to result in false positives, due to their specificity, the potential for false negatives is a great disadvantage. The GH_3 cell may be an appropriate *in vitro* screen for further development (Hohenwarter et al. 1996). Currently configured as a high throughput screen, it can identify TR agonists and antagonists. Based on evidence that several known thyroid disruptors that decrease serum TH levels have also been proposed as TR antagonists, additional research and characterization of the TR binding and activation of known thyroid disruptors is needed.

283. In conjunction with this effort, it may be useful to pursue computer-assisted screening of environmental chemicals for TR agonist and antagonist activity (i.e., *in silico)*. Schapira and coworkers (2003) have built a computer model of the antagonist-bound TR∀ ligand-binding domain, based on the crystal structure of the agonist-bound TR∀ ligand-binding domain (Darimont et al. 1998). Using this model, Schapira et al. (2003) predicted structures of TR∀ antagonists, then selected known compounds based on their structural similarity to the predicted models. These compounds were then tested for TR binding in cell culture. In addition, based on computer generated models, a small number of potential TR antagonists were synthesized and tested *in vitro* for TR binding. These authors confirmed that 14 of 75 compounds identified *in silico* could bind to the TR and antagonize TR action. Moreover, studying these 14 compounds *in silico* led to the identification of additional structures that were predicted to antagonize the TR. All were confirmed to act as TR antagonists *in vitro*. As more high quality data for the thyroid system is generated and can be incorporated into computer models, incorporation of virtual screening methods for both agonists and antagonists may be useful in identifying potential thyroid hormone disruptors within the vast array of environmental compounds.

5.0 THE HPT AXIS IN FISH AND ITS ROLE IN FISH DEVELOPMENT AND REPRODUCTION

[Editor's note: Some material in this section was taken from detailed review papers previously prepared for U.S. EPA on Fish Reproductive Screening Assays and on Partial Life Cycle Reproductive and Developmental Toxicity Tests. These materials have been included here because they are considered to be especially relevant to the purposes of this DRP.]

284. Among all the vertebrate groups discussed in this text, fish are the most diverse and demonstrate a high degree of heterogeneity in anatomy, physiology, reproductive strategy,[2] behavior, and ecology (Lagler et al. 1977; Janz 2000; Damstra et al. 2002). There are over 25,000 species of fish comprising six major groups: teleosts (e.g., bony ray fish, perch, bass); holosts (e.g., bowfin); chondrosts (sturgeon); chondricthyes (e.g., rays and sharks); cyclostomes (e.g., lampreys); and dipnoans (e.g., lungfish) (Lagler et al. 1977; Janz 2000). Taking into account all species of vertebrates, the fishes represent nearly one half (48%) of the species diversity; teleosts account for approximately 95% of that diversity (Lagler et al. 1977; Janz 2000). Accordingly, fish have evolved to inhabit a wide variety of environments in brackish, freshwater, and marine systems ranging from approximately 15,000 meters above sea level to 10,000 meters below (Lagler et al. 1977). The majority of research on the hypothalamus-pituitary-thyroid (HPT) endocrine axis in fish has been focused on teleost fish species, particularly salmonids and others that have economic value or that are readily available and easily cultured (e.g., cyprinids).

285. Thyroid hormones play an important role in the maintenance of a normal physiological state in vertebrates. It has been said that thyroid hormones influence the activity of a wider variety of tissues and biological functions than do any other hormones (Janz 2000). Thyroid hormones assist in control of osmoregulation, metabolism, somatic growth, development, and posthatching metamorphosis (Janz 2000). The control of metamorphosis in flounder and flatfish eye, mouth, and neural structures is associated with a dramatic spike in thyroxine[3] (T_4) concentrations (Janz 2000). Likewise, elevated levels of T_4 occur during smoltification in salmonids, reaching concentrations approaching 10 to 15 ng/mL (Janz 2000). These values represent some of the highest circulating T_4 levels observed in fish (Janz 2000). "Smoltification" is the process of transforming from a parr to a smolt. A "parr" is a young salmon actively feeding in fresh water. A "smolt" is a young salmon about two years old that is at the stage of development where it assumes the silvery color of the adult and is ready to migrate to the sea.

286. This chapter provides a review of the HPT axis of teleost fish. A thorough review of readily available scientific literature on the HPT axis was conducted. The HPT axis of fish differs, perhaps only slightly, from that of mammals. The features of these systems in fish will be reviewed with an emphasis on known differences in feedback relationships as well as differences in the molecular biology of the thyroid system in fish. This information will provide a tailored background for a review of the role of thyroid hormone in fish development and reproduction. Available information about the HPT axis in fish is detailed in some species and very limited in others. This review focuses on species that have been intensively studied, both because the information provides a stronger base from which to consider screens and tests for thyroid disruptors, and because these species are likely to be valuable laboratory models. Both *in vitro* and *in vivo* assays for screening chemicals for their potential to interfere with the HPT axis are reviewed.

[2]Fish can be oviparous, ovoviviparous, or viviparous.

[3]Thyroxine is specifically L-3,5,3',5'-tetraiodothyronine, abbreviated as T_4.

5.1 Overview of the HPT Axis

5.1.1 Central Nervous System and Endocrine System

287. In a broad sense, communication between cells and tissues can occur via the central nervous system (CNS) as well as through release of chemical messengers (hormones) or signals from the endocrine system.[4] Chemical signaling can be further divided into autocrine and paracrine actions to distinguish between effects on similar or different cell types. Although the focus in this chapter is the HPT axis of teleosts, the function of the endocrine system in general is much broader and contributes to the regulation of many physiological processes, such as digestion, metabolism, growth, and development. In essence, the endocrine system is involved with all phases of maintenance of homeostasis, and its function is intimately integrated with that of the CNS. Therefore, we initially describe the interaction between the CNS and thyroid system, and briefly discuss the reproductive system, as well, before describing control processes involved in regulation of these systems.

288. Neuroendocrine control of the HPT endocrine axis is exerted through actions of the brain. Both external and internal sensory information processed by the brain regulates secretion of hormones. Examples of external stimuli are temperature, photoperiod, and olfactory cues. Internal stimuli are represented by basal metabolism, growth, and chemical secretions from peripheral tissues such as gonads and sex steroids, for example.

289. In vertebrates, at least 10 different peptides and neurotransmitters can be formed by neurons within the hypothalamus (Bently 1998). Under proper stimulation, these hormones are secreted and in turn influence the release of pituitary hormones. The pituitary gland in fish, as in other vertebrates, consists of separate tissues called the neurohypophysis and adenohypophysis (Van Oordt and Peute 1983). The functional relationship between the hypothalamus and regions of the pituitary gland varies significantly among the different fish taxa, but in general, the evolutionary trend is toward increased control of pituitary function by neurological connections with the hypothalamus (Scott 1987). This corresponds to greater control by the pituitary of gonad development (Scott 1987). For example, teleost fish differ from other vertebrates in that a well developed portal blood supply between the hypothalamus and the adenohypophysis does not exist (Batten and Ingleton 1987; Peter et al. 1990). Rather, the adenohypophysis is directly innervated by neurosecretory fibers originating in the hypothalamus (Peter et al. 1990). Also in bony fishes, the blood flow to the adenohypophysis passes through the neurohypophysis (Scott 1987). The pituitary gland exerts control through secretion of several hormones, the most important of which with respect to reproduction are the gonadotropins, gonadotropic hormone-I (GTH-I) and GTH-II, and thyrotropin (TSH; Kime 1998).

290. The most important peripheral tissues involved in neuroendocrine control of reproduction are the gonads, which consist of the ovaries or testes. The thyroid system is also considered to aid in regulation of reproduction, although its specific role is less defined than that of the brain-pituitary-gonadal axis.

5.1.2 Thyroid System

291. The functional unit of the thyroid system in all vertebrates is the follicle, which consists of epithelial cells called thyrocytes. Thyrocytes enclose an extracellular space, forming a lumen into which they secrete a glycoprotein called thyroglobulin (Bently 1998). Thyroid follicles actively scavenge inorganic iodide from the blood, which is then incorporated into tyrosine residues within thyroglobulin. Thyroglobulin is successively oxidized by thyroid peroxidase enzyme to form L-thyroxine, commonly

[4] The endocrine system can be defined as any tissue or cells that release directly into the blood a hormone that signals or induces a physiological response in some target tissue (Thomas et al. 2001).

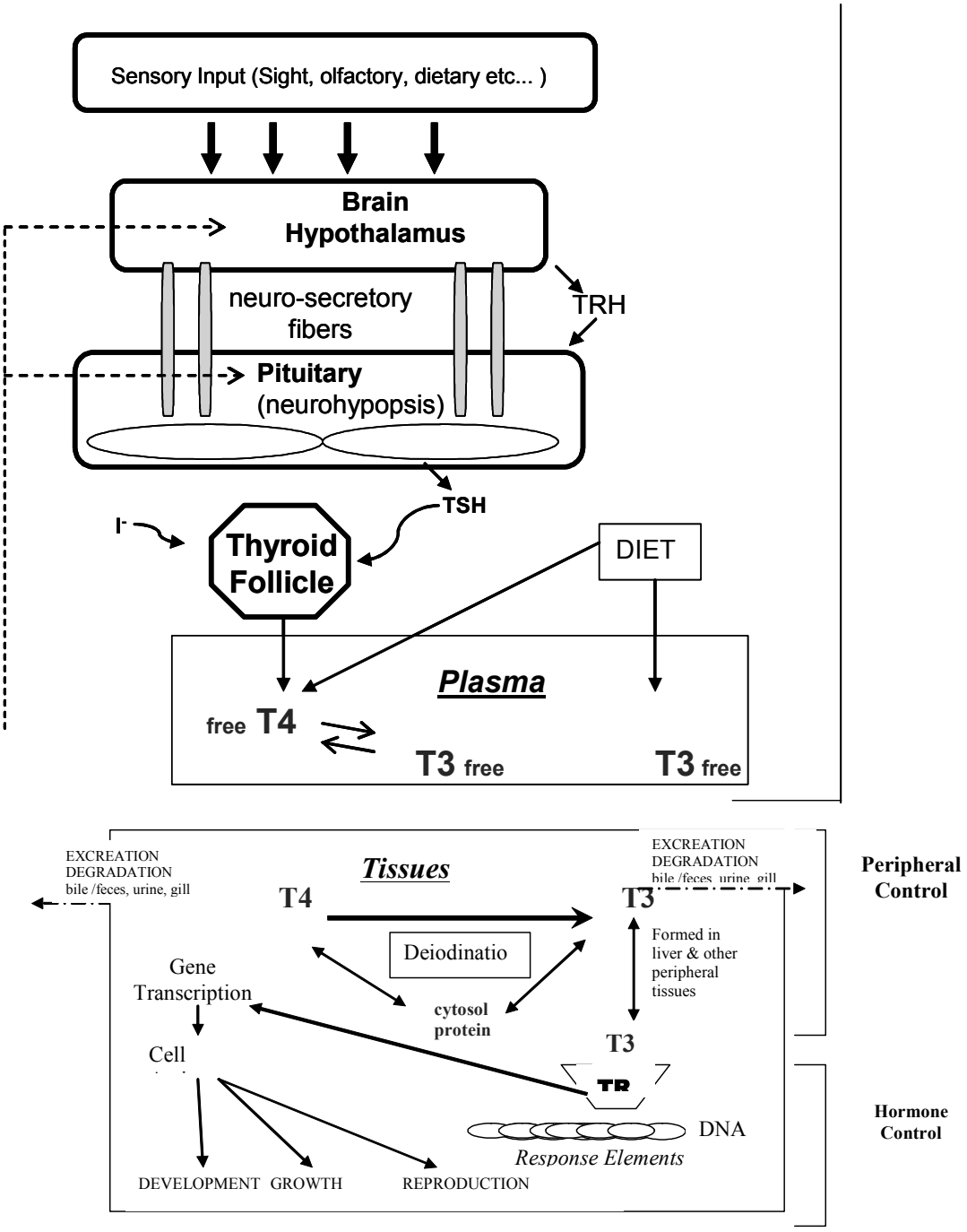
called T_4. T_4 synthesis is dependent upon the availability of free iodide; T_4 secretion is regulated by thyroid stimulating hormone (TSH) produced by the pituitary (Figure 5.1). While T_4 has long been recognized as an important thyroid hormone, it has more recently been considered a prohormone, required for production of biologically active 3,5,3'-triiodo-L-thyronine, commonly called T_3. The conversion of T_4 to T_3 occurs in peripheral tissue such as liver (among others). The T_3/T_4 ratios vary widely depending on physiologic state, including time of day, differences in salinity, reproductive condition, pH level, or during parr-smolt transformation in some species (Eales and Brown 1993).

5.2 Hormone Synthesis

5.2.1 Anatomy of the Adult Fish Thyroid Gland

292. The thyroid system in fish has been extensively investigated and reviewed, most thoroughly in the body of work by Eales and associated researchers who provide a detailed description of the system as well as describing development of assays to evaluate thyroid status in teleost fish (Eales 1979; Eales and Brown 1993; Bres et al. 1994; Eales et al. 1999). Although there are many similarities in the function and activity of fish and other vertebrate thyroid systems, there are also important differences. The fine structure of the fish thyroid exhibits greater heterogeneity than the mammalian thyroid, containing both follicles and cells of different sizes and functional states that are hypothesized to go through a histophysiological cycle of generation, maturation, and decay (Eales 1979). Eales (1979) recognized the importance of this heterogeneity and potential cycling to any future investigation into fish thyroid status or histology. Whereas the mammalian thyroid is usually a compact gland, the fish thyroid is more variable in form and location. It can be either compact or encapsulated with connective tissue or more commonly diffusely arranged around vascular tissue, (Eales 1979; Wendelaar Bonga 1993). The fish thyroid is also highly variable between and within fish taxa. Follicles can be dispersed in connective tissue near the pharyngeal region (e.g., in the fathead minnow, *Pimephales promelas*) (Wabuke-Bunoti and Firling 1983; Wendelaar Bonga 1993), located next to the ventral aorta (e.g., medaka, *Oryzias latipes*) (Raine et al. 2001), or can even migrate from the subpharynx to associate with the kidney (some freshwater cyprinids and poecilids). The epithelial cell height of salmon injected with bovine TSH for 4 days increases from about 8 to 10 μm to 20 μm (Nishioka et al. 1987).

Figure 5-1 Outline of the Brain-hypothalamus-pituitary-thyroid Axis, Peripheral Tissue Control and Hormonal Action (based upon Eales et al. 1999). **The output of thyrotropin (TSH) from the pituitary is regulated by neurochemicals from the brain, especially the hypothalamus. What these neurochemicals are is not clear. The pituitary TSH uniquely regulates the thyroid follicles, which are specialized in taking up iodide and iodinating tyrosines in the inert follicular colloid. The thyroid follicles in fishes are encapsulated in a gland in only a few species; in most fishes they are scattered around the throat. The fish thyroid releases primarily T_4, which is converted to T_3 by most, if not all, tissues. T_3 is typically more potent than T_4 in regulating gene transciption. In early life stages of some fish T_4 may be so abundant as to be the actual hormone that binds the thyroid hormone receptor (TR). There are multiple forms of the TR in species examined to date.**



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5.2.2 Thyroid Cascade

293. In teleosts the central control of thyroid hormone as depicted in Figure 5-1 is limited to the control of the production and secretion of T_4 (thyroxine). The biologically active thyroid hormone T_3 is derived from T_4 in the peripheral tissues, with emphasis placed upon the liver for extrathyroidal conversion (deiodination) (Eales et. al. 1999). In other words, increased synthesis of T_4 does not necessarily signal increased production of T_3 in the fish, so detected changes in T_4 may not indicate an adverse effect because T_3 function would remain the same. This is similar to the mammalian thyroid system, in which changes in circulating levels of T_4 do not always signal changes in tissue levels of the hormonally active T_3— especially in development. However, in fish, there may be less of an association between changes in serum T_4 and changes in tissue levels of T_3, and this is important in consideration of methods for measurement of thyroidal status in fish.

5.2.3 Regulation of Iodine Uptake

294. There are fundamental differences that exist in iodine (I^-) metabolism in fish compared to mammals. One primary difference is that fish have an extremely effective, highly vascularized gill surface with a branchial I^- pump that enables effective absorption of I^- from the water. Fish can obtain I^- from diet, but studies with brook trout in fresh water, that were starved for several weeks, were still able maintain elevated plasma I^- levels (Eales and Brown 1993 – citing Higgs and Eales 1971) demonstrating the effectiveness of the gill/branchial I^- pump, even in fresh water that has less I^- than salt water. In general, iodide levels in fish plasma are much higher than normal human levels (Eales and Brown 1993). Another significant difference between fish and mammals is the occurrence of a plasma "pre-albumin protein" (in some teleosts, e.g., clupeiforms) that actively binds to I^-; no such plasma iodide binding protein has been discovered in mammals (Eales and Brown 1993). For normal thyroid function the presence of I^- is essential; however, fish in natural and most artificial conditions do not suffer from having I^- deficiency (Eales and Brown 1993).

5.3 Hormone Release

295. Thyrotropin-releasing hormone (TRH) is a tripeptide that is highly conserved across all vertebrate groups (Bently 1998). In higher vertebrates, TRH functions to regulate pituitary release of TSH in addition to other pituitary hormones (Bently 1998). In fish, the functional role of TRH in regulating TSH release is less well established (Janz 2000). However, recent experiments using pituitary cells isolated from the bighead carp (*Aristichthys nobilis*) indicated that TRH exposure could upregulate TSH messenger RNA (mRNA) levels (Chatterjee et al. 2001). There have been two TRH pituitary receptors identified from fish; they are analogous to GnRH receptors in that they are cell-surface proteins (Harder et al. 2001). The two TRH receptors characterized in fish are structurally similar to those found in mammals (Harder et al. 2001).

296. In contrast to central nervous system control of sex steroid synthesis by the gonads, such as is exhibited with respect to GTH-I and II, thyroid hormone levels in fish are regulated to a much larger extent by peripheral tissues (Eales and Brown 1993). The functional activity of TSH is limited to regulating T_4 release and iodide uptake by the thyroid follicles (Eales et al. 1999). Secreted T_4 is converted to the active thyroid hormone, T_3, by an outer ring deiodination (ORD) process that is catalyzed by at least two different selenocysteine type, microsomal enzymes (Leatherland et al. 1990; Eales et al. 1999). In fish, ORD activity is typically highest in the liver, but is also present in other peripheral tissues (Darras et al. 1998; Eales et al. 1999). Only a single TSH receptor has been described in fish, which in some species is expressed only in thyroid tissue (Oba et al. 2001). In others, gonadal expression of a TSH receptor has

been reported (Kumar et al. 2000). The biological significance of gonadal expression of the TSH receptor is unknown. The action of T_3 on target cells in fish is also poorly understood (Cyr and Eales 1996).

297. Feedback control for thyroid hormone secretion is less complicated than steroid feedback actions and appears to be regulated primarily by a long feedback loop. In the few fish species studied (all teleosts), both T_4 and T_3 have a negative feedback effect on TSH secretion by the pituitary (Yoshiura et al. 1999). Consistent with findings for steroids, both T_4 and T_3 appear to decrease transcription of the beta subunit for TSH in the pituitary gland (Pradet-Balade et al. 1997; 1999). It is unknown whether T_4 or T_3 influences hypothalamic release of TRH; however, T_3 is known to decrease the synthesis of GnRH in tilapia (Parhar et al. 2000).

5.4 Conversion of T_4 to T_3

298. The conversion of T_4 to T_3 occurs via enzymatic removal (5′-monodeiodination) of the iodide component of the outer ring of T_4 (Eales et al. 1999). T_4 contains 4 iodine atoms. The removal of one of the 5′ iodine atoms from either of the two outer ring (phenyl) iodines results in the formation of T_3. As noted above, regulation of T_4 levels in plasma is the primary function of the brain-pituitary-thyroid axis in teleost fish as well as other vertebrates.

299. Thyroid hormones are activated and deactivated by deiodination, or stepwise removal of iodide from their outer or inner rings. However in fish, apparently more than in other vertebrates, important thyroid hormone transformations are controlled outside the thyroid (i.e., in the liver), and outer ring deiodination (T_4ORD) of T_4 to the more biologically active T_3 occurs mainly in peripheral tissue (liver, brain, kidney, gill) rather than in the thyroid itself (Figure 5.1) (Cyr and Eales 1988b; Mol et al. 1998; Bowen 1999; Eales et al. 1999). The availability of T_4 substrate is critical to T_3 production, but T_3 cycling is regulated differently in different peripheral tissues. Mol et al. (1998) and others report that the T_3 generated by the liver is usually exported to plasma, but that other tissues (brain, gill) containing T_3 receptors will bind T_3 and prevent it from entering plasma. T_4 can also be deactivated by inner ring deiodination (T_4IRD) to reverse T_3 (rT3, 3,3',5'-triiodothyronine) T_3 is degraded by inner ring deiodinase (T_3IRD) to 3,3'- triiodothyronine (T_2), which is presumed to be biologically inactive. In the few cases examined of salmon undergoing parr-smolt transformation, plasma and tissue T_3 levels are not correlated with plasma T_4 (Specker et al. 1992; Specker et al. 2000). A "parr" is a young salmon at the life stage from dispersion from the "redd" to migration as a smolt. The "redd" is the nest made by a salmonid comprising a hollow in the stream gravel, where eggs are laid, fertilized, covered with gravel, and oxygenated by the current. A "smolt" is a fully silvered (silver-colored) juvenile salmon during its first seaward migration.

300. Deiodination in mammals is accomplished by a family of type I, II, and III deiodinases (enzymes). Each active site has a genetically encoded selenocyteine, found in the endoplasmic reticulum (i.e., microsomal fraction) (Eales et al. 1999). In fish, deiodinases are similar to those in mammals; deiodinases contain selenocystein and are microsomal (Eales et al. 1999). However, the nomenclature of isozymes for fish does not use the type I, II, and III labels. The pathway of inner and outer ring deiodination is a simple enzymatically regulated pathway, controlled by the thyroid hormone substrate as described above. The enzymes responsible for T_4 outer-ring deiodination in trout are similar to type II enzymes in mammals; likewise the T_4 and T_3 inner ring deiodination enzymes in trout are similar to type III enzymes in mammals (Eales et al. 1999).

5.5 Thyroid Hormone Action

301. Accumulated information suggests that thyroid hormones variously affect growth, morphogenesis, skin pigmentation, osmoregulatory properties, and behavior in fish in general. For

example, such activity has been observed with parr-smolt phases of the coho salmon life cycle (Dickhoff, et al. 1978). The activity in the parr:smolt phases leads to the opinion that thyroid hormones may have a maturational role in developing fish, or in salmonids in particular, as the hormones do in developing amphibians (Dickhoff, et al. 1978). A number of researchers have investigated the role of thyroid hormone activity in fish embryogenesis, larval development, growth, and behavior, including relatively recent studies by Mol et al. (1997), Orozco et al. (1997), Specker et al. (2000), and Nayak et al. (2000). There have been very few studies on the direct role of thyroid hormone activity in fish reproduction (Leatherland 1982, 1994; Cyr and Eales 1996). Deiodination activity and rates in fish are responsive to many environmental and physiological conditions (e.g., food quantity, food quality, pH, salinity, turbidity) (Eales et al. 1999). The heterogeneity of fish thyroid systems and their resilience to perturbations make it difficult to measure and interpret changes in activity; the conclusion of Eales et al. (1999) that assays of fish thyroid function need to consider the multiple levels of the "thyroid cascade" is well-founded.

302. The thyroid hormones, T_4 and T_3, have been linked to a multitude of important functions in fish, such as growth, metabolism, and osmoregulation in addition to reproduction (Cyr and Eales 1996). Interference with thyroid hormone function could be expected to have wide-ranging effects on proper growth and development of gonadal tissues, and to affect estrogen synthesis in the ovary (Cyr and Eales 1988a, 1988b, 1989; Legler et al. 2000; Siwik et al. 2000; Soyano et al. 1993). Conversely, estradiol administration has been reported to lower circulating T_3 levels in immature trout (Alestrom et al. 1994). In the medaka, methyl testosterone (MT) exposure was reported to stimulate thyroid activity (Nishikawa 1976). These studies would suggest the possibility that sex steroid agonists might alter thyroid function.

303. Much research on thyroid function in teleosts has focused on salmonids. During the fish's upstream migration to spawning grounds, there is a decrease in both T_4 and T_3 plasma concentrations; however, thyroid serum levels tended to increase just prior to spawning (Cyr and Eales 1996). Such findings suggest that thyroid hormones may contribute to regulation of the initial stages of oogenesis in salmonid fish (Cyr and Eales 1996).

304. Despite these intriguing results, relatively few studies in fish have investigated whether environmental contaminants alter thyroid hormone status or directly interact with thyroid receptors to impair reproduction. However, one group of environmental contaminants, the polychlorinated biphenyls (PCBs), is of particular concern relative to thyroid hormones because of their structural similarity to one another. Studies in lake trout (*Salvelinus namaycush*) and a marine flatfish, the American plaice (*Hippoglossoides platessoides*), showed that exposure to selected PCB congeners can alter the turnover of T_4 and T_3 (Iwamatsu et al. 2000). Because PCBs affect thyroid hormone function in fish, a potential area of investigation might be to explore the mode of action of PCBs in the test species and to establish whether changes in the thyroid axis were coupled with endpoints of reproductive dysfunction. TSH activity is another research area that warrants additional study in the context of reproductive performance. Plasma levels of TSH are difficult to measure and rarely reported in fish reproductive studies, although the TSH receptor(s) might be expressed in oocytes and testicular cysts of some teleosts (Kumar et al. 2000).

5.5.1 Endpoint Sensitivity to Thyroid Stimulation

305. A search of the scientific literature revealed only one paper that addressed the effects of T_4 or T_3 exposure to a relevant test species. In this study, juvenile fathead minnows were exposed for 13 weeks to nominal water concentrations of 12.5, 25, and 50 µg/L T_3 (Abrahams and Pratt 2000). Exposure to 50 µg/L T_3 significantly decreased the growth rate of the minnows, although a clear dose-response relationship between T_3 exposure and growth could not be established (Abrahams and Pratt 2000). Although data on thyroid agonists are limited, it has been proposed that certain PCB congeners or their metabolites might bind to vertebrate thyroid receptors (Fentress et al. 2000; Brouwer et al. 1990). This potential would suggest that stimulation of the thyroid system is possible through a direct mode, such as

receptor-mediated action. However, the available data in fish regarding PCB exposure and thyroid status are contradictory (Schnurstein and Braunbeck 2001), and no generalizations can be made regarding the environmental significance of this mode of action.

306. Many studies in fish evaluate thyroid status by measuring circulating plasma T_4 and T_3 levels. A good example of this approach was a study of hydrogen cyanide exposure to rainbow trout reported by Ruby et al. (cited in Creech et al. 1998). Trout exposed for 12 days to 10 µg/L cyanide had significantly reduced E_2 and T_3 plasma concentrations, which corresponded to a lower gonadosomatic index (GSI) and oocyte diameters in females (Creech et al. 1998). Single measurements (or point estimates) of thyroid hormone levels might not be a good indicator of mode of action, because a complicating factor is the strong feedback control of T_4 and T_3 levels. For example, Adams et al. (2000) reported that a 5 µg/kg or 25 µg/kg intraperitoneal injection of PCB congener 77 lowered T_3 levels after 1 week in the American plaice. However, the same treatment stimulated conversion of T_4 to T_3 (specifically, T_4 outer ring deiodination) in liver microsomes (Schnurstein and Braunbeck 2001). These seemingly paradoxical results were hypothesized by the authors to be the result of increased clearance of T_3, which triggered the compensatory action of stimulating its biosynthesis by the liver (Schnurstein and Braunbeck 2001). A study of zebrafish exposed to ammonium perchlorate (a chemical used in rocket propellants, which is known to alter thyroid function in mammals by inhibiting the uptake of iodide by thyroid follicles, in turn inhibiting production of thyroid hormone) found that environmentally high concentrations of 18 ppm for 8 weeks affected the histological condition of thyroid follicles but did not impair reproductive performance. However, an exposure to 677 ppm for 4 weeks did impact reproduction, and it may have been due to extrathyroidal toxicity (Patino et al. 2003). These results illustrate the difficulty in assessing xenobiotic effects on thyroid status and the likelihood that point estimates of circulating thyroid hormones measured as part of a reproductive screen will not be useful by themselves in identifying thyroid disruption.

5.5.2 Inhibition of Thyroid Function

307. In contrast to the difficulties in identifying thyroid agonists, there is evidence that certain environmental contaminants can act specifically as antithyroidal agents. A thorough study of the effects of thiocyanate (an inorganic anion that has antithyroidal properties) on thyroid function and reproduction in fathead minnows was reported by Lanno and Dixon (1990, 1994). In these studies, sexually differentiated but immature fathead minnows were exposed to measured concentrations of thiocyanate ranging from 0.06 mg/L to 32.6 mg/L for 21 days and then for an additional 103 days, during which spawning activity was monitored. The results indicated that fathead minnows exposed to 16.6 mg/L and 32.6 mg/L thiocyanate completely lacked or underwent incomplete development of secondary sex characteristics. These fish also made no attempt to reproduce (Lanno and Dixon 1994). However, toxicity was quite high at these exposure levels, with reported mortalities during the exposure of 30% and 63% at the 16.6 mg/L and 32.6 mg/L exposure levels, respectively (Lanno and Dixon 1994). A lower exposure rate of 7.3 mg/L was nontoxic but still impaired reproduction, as measured by the delay in time of first spawning and decreased fecundity (Lanno and Dixon 1994). Antagonism of thyroid function was observed at 7.3 mg/L to 32.6 mg/L thiocyanate exposure rates by the development of overt goiterous nodules along the branchial region of the lower jaw. Histopathological examination of these fish indicated a clear dose-response relationship between thiocyanate exposure rate and the extent of the hyperplastic and colloidal goitrous follicles (Lanno and Dixon 1994). The time to first appearance of the goiters or gender-specific differences of goiter formation were not discussed in this study.

308. Consistent with the findings of the aforementioned study, other antithyroidal agents have been shown to cause hyperplasia of thyroid follicles and decreased development of secondary sex characteristics. For example, adult catfish (*Clarias batrachus*) exposed for 1 year to 100 mg/L ammonium sulphate or 3 months to 300 mg/L thiourea, which are known anti-thyroidal agents (inhibitors of thyroid

hormone syntheses) (nominal levels, respectively) developed complex histopathology of the thyroid follicles, suggestive of overall hyperemia and hyperplasia (Sathyanesan et al. 1978). In zebrafish exposure to perchlorate for 12 weeks caused colloid depletion, hypertrophy of the thyroid epithelium, angiogenesis around the follicles, and depletion of colloidal T_4 in the ring surrounding the lumen; however, whole-body T_4, body growth, and condition factor were not affected (Mukhi et al. 2005). This indicates that several aspects of thyroid histology are sensitive indicators of antithyroidal agents. In medaka, an intraperitoneal injection of thiourea reduced the microtubule-stimulated growth of papillary processes of the anal fin (Fujiwara 1980). In a related study, Wester et al. (1988) noted that medaka exposed for several months to various sodium-bromide water concentrations had reduced secondary sex characteristics. A study in sexually mature medaka measured T_4 and T_3 plasma levels before and after a 10-day exposure to various nominal water concentrations of thiourea (Tagawa and Hirano 1991). Exposure to 300 mg/L thiourea lowered plasma levels of T_4 and T_3 from 8 ng/mL and 5 ng/mL respectively, to less than 2 ng/mL within 24 hours. A similar reduction in thyroid hormones was observed in eggs laid by exposed females. The effects of thiourea exposure on fecundity were not reported; however, fertilization success and time to hatching were unaffected by the exposure (Tagawa and Hirano 1991). Larval survivability was also unaffected by the thiourea exposure. Immersion of larval flatfishes in 30 mg/L thiourea inhibited settling behavior, survival, resorption of the dorsal fin ray, stomach formation, gill formation, metamorphosis as indicated by translocation of the eye, and seawater tolerance (Miwa and Inui 1987; Miwa et al. 1992; Huang et al. 1998; Schreiber and Specker 1998; Schreiber and Specker 1999a,b,c; Gavlik et al. 2002).

5.5.3 Gender Differences

309. Due to the paucity of studies available for review, little discussion of gender differences in thyroid stimulation or inhibition can be made. Certainly, more research is needed that focuses on thyroid agonists or direct stimulation by T_3 and T_4 and subsequent effects on reproduction. The limited data on antithyroid compounds suggest that both male medaka and fathead minnows might be more sensitive models. In these species, impairment of the appearance of secondary sex characteristics, such as papillary processes on the anal fin in medaka (Fujiwara 1980) or nuptial tubercles in minnows (Lanno and Dixon 1994), might become apparent after short-term exposures. For example, exposure to 16.6 mg/L of thiocyanate resulted in incomplete development of secondary sex characteristics; fish exposed to lower concentration of 7.3 mg/L demonstrated antagonism of thyroid functions by development of overt goitrous nodules along the branchial region of the lower jaw. Histopathology of these fish indicated dose-response relationships between the thiocyanate exposure rate and the extent of the hyperplastic and colloidal goitrous follicles (Lanno and Dixon 1994). However, it is unclear whether significant goiter formation can occur in these species over the relatively short exposure periods (14 to 21 days) used in screening assays. In this respect, histopathological analysis of the thyroid follicles would be particularly helpful in identifying antithyroidal chemicals.

310. The limited data on gender differences serve to underscore the broader issue of the scarcity of information on basic thyroid function in fish. Clearly, more basic information on thyroid function during reproduction in the typical test species is needed. Therefore, it is prudent that any endocrine disruptor screening assay should include some measure of effect on thyroid tissue. However this may not be a simple task for most fishes, because the basic unit of thyroid tissue, the thyroid follicles, are typically distributed in a diffuse manner throughout the ventral pharyngeal region and sometimes at secondary locations within the fish, especially the head kidney, but also the ovary, head, kidney, and pericardium (Wendelaar Bonga 1993; Janz 2000). The dispersed nature of thyroid tissue in fish makes certain morphological assessments of thyroid tissue, such as determination of a thyroid tissue-somatic index, difficult. However, other assessments of thyroid tissue such as vascularization, epithelial cell height, and the colloidal T_4 ring, which is T_4 immunoreactivity at the interface between the follicular epithelium and the colloid, all respond significantly in zebrafish exposed to perchlorate for 12 weeks (Mukhi et al. 2005).

The lowest-observed-effective concentrations (LOECs) were 90, 1131, and 11 ppb, respectively for each of the tissue assessments listed. Further work on the colloidal T_4 ring is merited.

5.6 Thyroid Hormone Metabolism

311. Studies have shown that fish can excrete relatively large quantities of injected T_4 and T_3 via the bile (Eales 1979). The conjugates of T_3 occur principally in the liver followed by the excretion via bile of the water soluble fraction. To a limited extent, unconjugated T_4 and T_3 can be absorbed from the intestine (Brown et al. 2004). Excretion of T_4 and T_3 to a lesser extent can also occur through gill surface (Brown et al. 2004).

5.7 The Role of TH in Fish Development and Reproduction

5.7.1 Thyroid Hormones in Fish Eggs and Larva

312. Thyroid hormones in mammals, birds, and amphibians play an important role in early development. The role of TH in developing oocytes and the absolute requirements of thyroid hormone are not well known. In comparison to what is known about the effects of thyroid hormones on embryos and larvae of amphibians, mammals, and birds, our knowledge regarding fish lags behind. Studies using medaka *(Oryzias latipes)* (Tagawa and Hirano 1991) and rabbitfish (Ayson and Lam 1993) demonstrated that reduction in egg thyroid concentration did not adversely impair larval development. A review paper by Raine et al. (2002) on thyroid gland development states that only three papers have been published since the late 19th Century on "early ontogeny of the thyroid tissue of teleost fishes" (authors cite Maurer, 1886; Hoar 1939; and Raine et al. 2001). Thus, detailed information on the formation of thyroid follicles in embryos is lacking. Histological investigation by serial sectioning in rainbow trout revealed that in late embryogenesis, post 40 days fertilization (at 8C water temperature), distinct thyroid follicles become apparent. Measurable concentrations of T_4 and T_3 in eggs of Chinook salmon *(Oncorhynchus tshawytscha)* were 4.2 and 4 ng/organism respectively (Leatherland et al. 1989). In sockeye salmon *(Oncorhynchus nerka)* the concentration of T_4 and T_3 in eggs was 6 and 1 ng/organism respectively (Leatherland et al. 1989). Tagawa et al. (1990) measured T_4 and T_3 in eggs of 26 species of fish with the mean T_4 and T_3 concentration ranging from 15.00 ng/g (chum salmon) to 0.04 (marbled sole) and 9.95 ng/g (Pacific herring) to 0.07 (goldfish) respectively. In general the concentrations of T_4 are higher than those of T_3 in freshwater species; however sometimes T_3 is higher in marine species (Power et al. 2001; Tagawa et al. 1990). In newly-hatched coho salmon (Oncorhynchus kistuch), the yolk alone constitutes 90% of the total weight and contains 96% of the total T_4 (Kobuke et al. 1987). The T_4 decreases during embryonic and larval stages and first increases at the time the coho salmon become free-swimming active feeders. Because thyroid follicles are not present in eggs, the origin of T_4 and T_3 in fish eggs is maternal (Power et al. 2001). It is still unclear if the thyroid hormones in eggs are available to the developing embryo (Power et al. 2001). A slight decrease in thyroid hormones was observed in eggs of seven fish species just prior to hatching; however, a significant decrease was observed during yolk absorption in flounder, coho salmon, and chum salmon (Kobuke et al. 1987; Tagawa et al. 1990). In a study with medaka (exposed to thiourea), where maternal thyroid hormone content was altered such that a 90% reduction of thyroid hormone content in eggs was achieved, no discernable difference was observed in hatchability and time of hatching (Tagawa and Hirano 1991). In addition, no difference was observed between control and thyroid limited eggs in survival rate, body length, weights, and condition factor post 16 days (with food supplied) after hatch out (Tagawa and Hirano 1991). This led the authors to conclude that the majority of thyroid hormone in medaka eggs was not essential for larval survival and development. However, it may be possible that less than 10% of thyroid hormone in eggs is required for normal development.

313. Studies have demonstrated that larval teleosts respond when exposed to exogenous T_3 at very high doses for weeks at a time. In such a setting the general response has been enhanced growth, survival,

and time of yolk sac absorption (Brown et al. 1987). In larval flounder, immersion in 0.1 ppb T_3 for one month accelerated the rate of settlement, whereas immersion in 1 ppb T_3 for one month accelerated shortening of the dorsal fin ray (Miwa and Inui 1987).

314. The development of the thyroid has been described in a small variety of teleosts (see Pickford 1957; and below). In the Nile tilapia, at the yolk sac larva stage there are no thyroid follicles, whereas they appear later in the fry stage (Nacario 1983). Histological changes occur in the thyroid follicles of metamorphosing larval flounder (Miwa and Inui 1987). In fact, in flounder sampled 36 h after hatching, T_4 was detected in the subpharyngeal region by immunocytochemistry. In 4-day-old flounder a few thyroid follicles were present. The number and size of follicles increased from premetamorphosis to prometamorphosis and maximal epithelial cell height occurred at metamorphic climax. Histological investigations of larval striped bass have shown that thyroid follicles become functional at approximately 3 weeks of age, with increased activity occurring from week 3 through week 6 (Brown et al. 1987).

5.7.2 Thyroid Hormones in Fish Larva and Adult

315. As previously mentioned, fish are a very diverse group; many undergo an indirect developmental progression from embryos, to larvae to juveniles, and adults; however, others undergo direct development from the embryonic stage to the juvenile. The larval stage can be distinct both morphologically and physiologically from the juvenile stage. The transformation from larval stage to juvenile is termed metamorphosis when it includes an irreversible change in body form and a habitat shift (Youson 1988). Thyroid hormones are essential is this transformation processes. The most striking example of this occurs in flatfishes (flounder, halibut) in which a bilaterally symmetric pelagic larva transforms to an asymmetric benthic juvenile that has both eyes on the same "up" side of the fish. Thyroid hormones are necessary and sufficient for this process and increased whole-body T_4 occurs during metamorphic climax. In addition to distinct external morphological changes that occur during larval metamorphosis, internal alterations are occurring. For example, often in teleosts a functional stomach is not developed until the larval-juvenile metamorphosis is completed (Tanaka 1971). In "symmetrical fishes," external morphological changes are not as dramatic as in the asymmetrical flatfishes and involve changes in fin structure, scaling, lateral line formation, and pigmentation. Studies on grouper (deJesus et al. 1998) and zebrafish (Brown 1997) have demonstrated that exogenous THs were found to accelerate pelvic fin growth and induce early differentiation of pectoral fins. Conversely, exposure to thiourea (goitrogen–thyroid synthesis inhibitor) inhibited the larval to juvenile metamorphosis in zebrafish (Brown 1997). However, in other teleosts such as lamprey (agnathan) exposure to thiourea induced early metamorphosis (Holmes and Youson 1993). It is apparent that the THs play an important role in larval to juvenile metamorphosis. However, much information is lacking on specific modes of action, and the manner by which THs bring about their effect is less clear in fish than in other vertebrates.

5.8 Methods of Evaluating Thyroid Disruption in Fish

316. A number of researchers have investigated the role of thyroid hormone activity in fish embryogenesis, larval development, growth, and behavior, including relatively recent studies by Mol et al. (1997), Orozco et al. (1997), Specker et al. (2000), and Nayak et al. (2000). There have been very few studies on the direct role of thyroid hormone activity in fish reproduction; one significant review on the subject is provided by Cyr and Eales 1996, entitled "Interrelationships between thyroidal and reproductive endocrine systems in fish"). Deiodination activity and rates in fish are responsive to many environmental and physiological conditions (e.g., food quantity, food quality, pH, salinity, turbidity) (Eales et al. 1999). The heterogeneity of fish thyroid systems and their resilience to perturbations make it difficult to measure and interpret changes in activity. Eales et al. (1999) conclude that assays of fish thyroid function need to consider the multiple levels of the "thyroid cascade" by measuring components of the central control

(Brain-pituitary-thyroid) and peripheral control of T_3 production and metabolism (such as the liver), in addition to measurements of post receptor-mediated effects of T_3 on target cells.

5.8.1 Overview of Experimental Methods

317. An Environmental Protection Agency (EPA)-sponsored workshop in 1997 concluded that all known chemicals that interfere with thyroid hormone action, function, and homeostasis act by inhibiting synthesis of thyroid hormones, altering serum binding to transport proteins, or increasing thyroid hormone metabolism (DeVito et al. 1999).

318. The thyroid systems of fish and mammals are similar in many respects, with one major difference. The mammalian system is driven primarily through the central brain-pituitary-thyroid axis that regulates thyroidal secretion of both T_4 and T_3. This central control, which includes strong feedback by T_3 on the brain-pituitary-thyroid axis, allows the effects of a xenobiotic (e.g., erythrosine) on the peripheral metabolism of thyroid hormone to be detected through a change in thyrotrope function and thyroid stimulating hormone (TSH) release (Eales et al. 1999). Thus, in mammals (i.e., rodents), TSH secretion is commonly used to assess risk to thyroid function at both central and peripheral levels.

319. In fish, the thyroid system does not appear to be driven primarily by the central brain-pituitary-thyroid axis. Instead, the central brain-pituitary-thyroid axis in fish has the primary role of ensuring T_4 homeostasis. T_3 production and homeostasis is regulated in peripheral tissue by conversion of T_4 to T_3 by deiodination, removal of either the inner or outer ring iodide from T_4 (refers to the deiodination, or stepwise iodine removal, from outer or inner rings of T_4) (Eales et al. 1999). In teleost fish the routine role of the central brain-pituitary-thyroid axis may be to ensure T_4 homeostasis, so as to provide an adequate supply of T_4 prohormone to satisfy peripheral demands for T_3. Eales et al. (1999) conclude that "This difference in control emphasis (peripheral versus central) between the fish and mammalian systems has important implications for measurement of thyroidal status in fish."

320. The implication is that no single biomarker examines all facets of fish thyroid function. Xenobiotic effects on fish thyroid function have typically been assessed from changes in TH biosynthesis or TH secretion or plasma TH levels. Because these indices relate mainly to the efficacy of TH release from the thyroid to the blood, they do not necessarily detect disruption of TH metabolism or receptor and post-receptor TH effects in peripheral tissues (Eales et al. 1999). To screen for xenobiotic effects in fish requires examination at three levels: 1) the centrally controlled thyroidal secretion of T_4 prohormone to the plasma (T_4 homeostasis), 2) the peripherally controlled conversion of T_4 to active T_3 (T_3 homeostasis), and 3) the post-receptor effects of T_3 (Eales et al. 1999). Even then, because of extensive autoregulatory feedback at both the central and peripheral levels, overall euthyroidism (T_3 availability to receptors) may be preserved despite potentially disrupting xenobiotic effects on T_4 and T_3 homeostasis. Thus, current assays of thyroid function represent primarily biomarkers of exposure and not necessarily biomarkers of effect.

5.8.2 Whole Animal Assays

321. Thyroid status and function have been studied in approximately 50 species of teleosts (Cyr and Eales 1996). In these studies various measurement endpoints have been employed, such as histological appearance of thyroid tissue or pituitary thyrotropes; measurement of radioiodide or protein-bound iodine levels in plasma; measurement of plasma T_4 or T_3 levels by radioimmunoassay; measurement of thyroid hormone receptor levels; and measurement of thyroidal protease activity, etc. However, performance of these measurements alone does not necessarily constitute development of an assay screen or test suitable for detection of thyroid hormone disruption in fish. An example of morphological measures of metamorphosis and thyroid status includes resorption of dorsal fin rays and migration of the eye in

Japanese flounder *(Paralichthys olivaceus)*. Resorption of the dorsal fin rays in the Japanese flounder have been likened to the resorption of the tadpole tail in amphibian metamorphosis (deJesus et al. 1990). During Japanese flounder metamorphosis from bilaterally symmetrical larvae to asymmetrical juveniles, the dorsal fin ray elongates and is subsequently resorbed. Inui and Miwa (1985) showed that exogenous thyroid hormone accelerates metamorphosis in the flounder. The thyroid hormones influenced the metamorphic process in a dose-dependent manner, with T_3 proving several times more potent than T_4 (Miwa and Inui 1986). It is apparent that metamorphosizing flounder respond to thyroid hormones, and thyroidal influence on metamorphic events in the flounder involve the alteration or initiation of synthesis of tissue-specific proteins such as myosin, troponin T, pepsinogen, and hemoglobin (Yamano et al. 1994). However, it is unknown how TH regulates tissue development and differentiation, including synthesis of tissue-specific proteins (Yamano et al. 1994).

322. As an example of a whole animal fish assay, prometamorphic larvae of Japanese flounder were placed in tanks with different concentrations of T_4 (0.0 to 0.10 µg/ml) (deJesus et al. 1990). Hormone levels were kept constant, and 20 percent of the water was exchanged daily. Fish were fed, and every 5 days a subset of fish were randomly sampled and body length and length of the second fin ray was measured. The degree of eye migration was also estimated following the methods of Miwa and Inui (1987). Protocols ultimately developed to morphologically mark thyroid impairment in fish (parr-smolt transformation; young fish metamorphoses) may include any applicable endpoint and should not be limited to one endpoint due to the complexity of the fish thyroid cascade (central and peripheral control).

323. There are a limited number of routes of exposure of fish to endocrine disruptor compounds. They most often include water, but also include oral and parenteral exposure. Typical practical considerations for sample size are based on the number of endpoints to be collected and whether the specimen must be sacrificed to collect the data. In order to statistically determine the appropriate sample size, the inherent variability of the endpoint must be measured, according to the desired statistical resolution, and the power of predictability determined. The length of the prospective assays and the natural variability associated with metamorphosis, smoltification, or other change may require larger sample sizes than used for short-term partial life-cycle tests. As a useful guide, 100 fish larvae per replicate has been a standard sample size for starting a long-term exposure for regulatory purposes. However, before sample size and replicate requirements can be determined for the fish assays, formal statistical power analysis is required.

324. A larval fish assay concept was put forward by Brown et al. (2004, SETAC Journal). The authors conclude that "Young developing fish largely have been ignored in studies to date but may be particularly susceptible to thyroid disruption and should be a focus of future work. Molecular biological techniques should be applied to thyroid analyses of extremely small fish." Larval fish would be an area worthy of further study. Many of the observations regarding the flounder assay are applicable to the larval fish assay.

325. Augmented thyroid hormone levels may initiate changes in the functional activity of the interrenal tissue. Interrenal cells have been studied during Japanese flounder metamorphosis (deJesus et al. 1991). Histological examination indicated an increase in the size and number of interrenal cells during metamorphosis. The thyroid gland also showed signs of activation. Japanese flounder interrenal tissues were fixed in Bouin's fixative, then dehydrated with a graded series of ethanol, embedded in Paraplast, and sectioned to 4 µm thickness. Sections were stained with hematoxylin and eosin.

326. Histological observations in Atlantic salmon (*Salmo salar*) and Pacific salmon (*Oncorhynchus* spp.) also indicate that the pituitary-interrenal axis is activated during smoltification (Specker and Schreck 1982). Further experimental work would be required to confirm any action of thyroid hormones on interrenal tissue of larval or juveniles fishes.

5.8.3 In Vitro and Ex Vivo Assays

327. Biochemical measurement of thyroid activity can be measured in plasma obtained from cardiac puncture and whole body tissue. TH (T_3 and T_4) and deiodinase activities have been analyzed in fish primarily using radioimmunoassay (RIA) methods. Regardless of method, quality assurance (QA) measures associated with RIA analyses should include an evaluation of cross-reactivity with other hormones or similar substances, evaluation of linearity using standard curves, and the use of standard additions to assess recoveries.

5.8.3.1 Plasma TH Levels

328. Because the structures of T4 and T3 are the same in fishes as in mammals and other vertebrates, it is easy to use commercially-available antiserum in radioimmunoassay and enzyme-linked immunoassays. This is an advantage when aiming to quantify TH. In practice, TH can be measured in plasma and also in extracted tissues. Because larval fish are very small, their whole-body can be homogenized and extracted for TH content. The following radioimmunoassay method for measuring plasma L-thyroxine (T_4) levels is taken from Dickhoff et al. (1978), who measured TH levels in the blood of coho salmon *(Oncorhynchus kisutch*). This method is often cited, with minor modifications, in other studies that measure whole-body TH levels in larval fish. For example, thyroid hormones of the Japanese flounder (*Paralichthys olivaceus)* and summer flounder *(P. dentatus)* were extracted and measured by radioimmunoassay (RIAs) following the method of Tagawa and Hirano (1989). The hormones from both newly fertilized eggs and larvae were evaluated to determine changes over time during early development and metamorphosis of this species (de Jesus et al. 1991; Schreiber and Specker 1998). Likewise, T_4 and T_3 serum concentrations were measured in common dentex (*Dentex dentex)* to investigate seasonal changes in serum levels of thyroid hormones during the first, second, and third reproductive cycles and to determine possible correlations with growth patterns, gonadal development, and spawning (Pavlidis et al. 2000). Commercialized kits were used to analyze the T_3 and T_4 thyroid hormones using coated tube radioimmunoassay methods with slight modification to the procedure. Once baseline conditions are established for various fish species during different life stages and under standardized holding conditions, researchers can examine whether differences occur when fish are exposed to chemicals that disrupt thyroid hormone homeostasis or thyroidal status. A primary disadvantage to measuring TH in fishes is the diurnal and seasonal hormonal rhythms which occur. Special care would have to be imposed to ensure that these natural fluctuations in TH titers of experimental fishes are appropriately controlled.

329. In the Dickhoff et al. (1978) method, plasma samples were collected from yearling coho salmon. To obtain the plasma samples, unanesthetized fish were stunned by a sharp blow to the head and the tail amputated. Blood was collected in a heparinized capillary pipet. The blood was centrifuged and the plasma stored at -20˚C in plastic microcentrifuge tubes until assayed. A thyroxine RIA was performed using antiserum and high specific activity (700 mCi/mg) ^{125}I-labled thyroxine. For the assay, 10-µl aliquots of plasma were added in duplicate to the assay tubes. To these tubes containing plasma or T_4 standards, 250 µl of the following mixture was added:

Compound	**Volume**
Bovine γ -globulin	150 mg
8-anilino-1-naphthalenesulfonic acid (sodium salt)	60 mg
Radioactive T_4	12 X 10^6 cpm
0.11*M* barbital buffer (pH 9.0)	100 ml
Antiserum	N/A

330. Antiserum was previously diluted to a concentration that resulted in 50% labeled T_4 bound with no added unlabeled T_4. Tubes were capped and incubated for 30 minutes at 37˚C followed by 15 minutes at 4˚C. Antibody was then precipitated by adding 0.3 ml cold (4˚C) 20% (w/v) polyethylene glycol followed by thorough mixing. The precipitate was centrifuged at 2000g for 15 minutes at 4˚C. The supernatant was then aspirated and the pellet was counted in a gamma well counter for 3 minutes/tube. Dilution of coho plasma showed parallel cross-reactivity with the T_4 standard, but treatment of coho plasma with an equal volume of dextran-coated charcoal (5 g/L or Norit A and 5 g/L of dextran) removed all immunocross-reactivity.

5.8.3.2 Deiodination Assay

331. The peripherally controlled conversion of T_4 to T_3 cannot be monitored reliably from plasma T_3 levels alone. However, deiodination activities (e.g., *in vitro* assessments of a suite of rate-limiting deiodinations in the liver and brain) should adequately evaluate peripheral thyroidal (T_3) status. Eales et al. (1999) propose deiodination assay methods that could be used as sensitive indices of peripheral change and as biomarkers of exposure. The advantages of these measures of deiodinases are that they are sensitive, they reflect end-target use of TH, and the components can be obtained commercially. The disadvantages of deiodinase assays are that they require fairly good technical skills (HPLC), they involve proper handling and disposal of radioactive isotopes, and, because the ^{125}I has a short half-life of 60 days, samples cannot be stored once processing begins.

332. Eales et al. (1999) includes a T_4ORD (outer ring deiodination) assay for estimating tissue T_4 to T_3 conversion, and an assay run simultaneously that determines T_4IRD activity by measuring $*rT_3$ by HPLC (* indicates a radioassay, conducted with [^{125}I]). T_3 ORD and IRD activity is measured in a similar manner, but substituting $*T_3$ as a substrate in place of $*T_4$ in the appropriate part of the assay. After T_3 is formed by removing one of the outer ring iodines from T_4, the generated T_3 may be measured directly by RIA following *in vitro* incubation of T_4 substrate with either tissue homogenate or subcellular fractions. An even more sensitive approach uses a radiolabeled ([^{125}I]T_4, or $*T_4$) substrate and measures either the $*I^-$ or the $*T_3$ products. The assay is preferably performed on the deiodinase-rich microsomal fraction, which may be prepared from fresh tissues or partially thawed tissues previously quick-frozen in liquid nitrogen. The deiodination rate is then calculated as pmol T_4 diodinated per hr per mg microsomal protein from the total concentration of $*T_4$ substrate, the amount of $*I^-$ or $*T_3$ generated and the microsomal protein content.

333. T_3 production in rainbow trout has been measured directly using a similar RIA following *in vitro* incubation of T_4 substrate with tissue homogenate or subcellular fractions. A more sensitive approach is to employ outer-ring labeled [^{125}I] T_4 ($*T_4$) substrate and then measure the levels of $^{125}I^-$ ($*I^-$) and/or [^{125}I] T_3 ($*T_3$) produced in theoretically equivalent amounts (Bres et al. 1994). The products can then be quantified by HPLC (Sweeting and Eales 1992).

5.8.3.3 Isolation of Thyroid Hormone Receptors

334. Thyroid hormone-activated thyroid hormone receptors (THRs) bind directly to a *cis*-acting element of a gene to regulate expression of the gene either positively or negatively. Thus, it could be expected that the localization of THR during fish metamorphosis could provide key information about target tissues for TH and TH-inducible protein synthesis during metamorphosis (Yamano et al. 1994). Homologous probes or antibodies for fish THR are not common, although they are available for mammals, chickens, and frogs. To know how similar or dissimilar the fish THRs are from those of other species, isolation of cDNAs for THRs expressed in fish are needed. Two Japanese flounder THRs were identified by Yamano et al. (1994). Both were considered to be α-type THRs rather than β-type. The flounder appears to have two distinct genes for two THRs, making it more similar to the amphibian, *Xenopus*, than to mammals. The binding properties of these flounder THRs to TH or other TH-responsive elements had

not yet been determined (Yamano et al. 1994). Two β-type THRs were later identified in the flounder as well, though they were found to be encoded by a single gene (Yamano and Inui 1995). Thus, at least three THR genes generate at least four THR proteins in the flounder genome (Yamano and Inui 1995).

5.8.3.4 Thyroid Hormone Receptor Assay

335. The following method was used by Bres et al. (1994) to study properties of TH nuclear receptors in rainbow trout *(Oncorhynchus mykiss)* tissues. It is an *in vitro* method that employs the principle of saturation analysis and may be conducted on intact nuclei from liver, although nuclei from other tissues are unstable and the receptor must be solubilized and extracted for the assay. The advantage of this method is that it uses classical reversible bimolecular binding as the model for determining receptor sites, and it could be utilized as a screen to identify chemicals that have the potential to interact with the thyroid receptor(s) that is tested. The disadvantages are that the saturation kinetics need to be determined for each species and each tissue, and that the assay is technically challenging. Further, the relationship between thyroidal system status (active, inactive) and receptor number is not clear, and such an assay would only capture one potential site of disruption within the thyroid system.

336. Bres et al. (1994) used the following general binding assay method: the whole nuclei or solubilized receptors are incubated to equilibrium with $*T_3$ in the presence of different concentrations of T_3. The receptor-bound and the free $*T_3$ are then separated by either low-speed centrifugation (for whole nuclei) or by using Dowex ion-exchange resin, that binds "free" $*T_3$ (for solubilized receptors). Specific binding to the receptor can be distinguished from nonspecific binding (i.e., $*T_3$ trapped within the nuclear pellet or bound to the walls of the assay tube) by adding a large excess of unlabeled hormone at a level about 1000 times the Kd of the receptor. In this circumstance, the receptor-bound $*T_3$ is released and there is a reduction in bound counts. The $*T_3$ that remains bound (nonsaturable binding) is subtracted from the total counts bound:

Saturable binding = total binding – nonsaturable binding.

337. The units of each term are in counts per minute (cpm) or in moles of $*T_3$. The saturable binding is considered representative of specific binding to the receptors. Detailed methods are found in the literature (Eales 1999).

338. Using the reversible bimolecular binding model, the methods outlined in the report can be used to calculate the receptor properties, including the binding affinity, capacity, specificity, and the association and disassociation rate constants. Furthermore, the procedures described may be adapted to study aspects of receptor function *in vivo*. One caveat the Bres et al. (1994) paper makes is that the methods described apply to the rainbow trout. Because the conditions have been found to differ even in different tissues of this species, the authors recommend that incubation times and temperatures be experimentally determined in any new species or tissue, and that the incubation times be long enough for binding equilibrium to be achieved.

5.8.3.5 Ex Vivo Assay - Thyroid Hormone Effects on Fin-ray Resorption

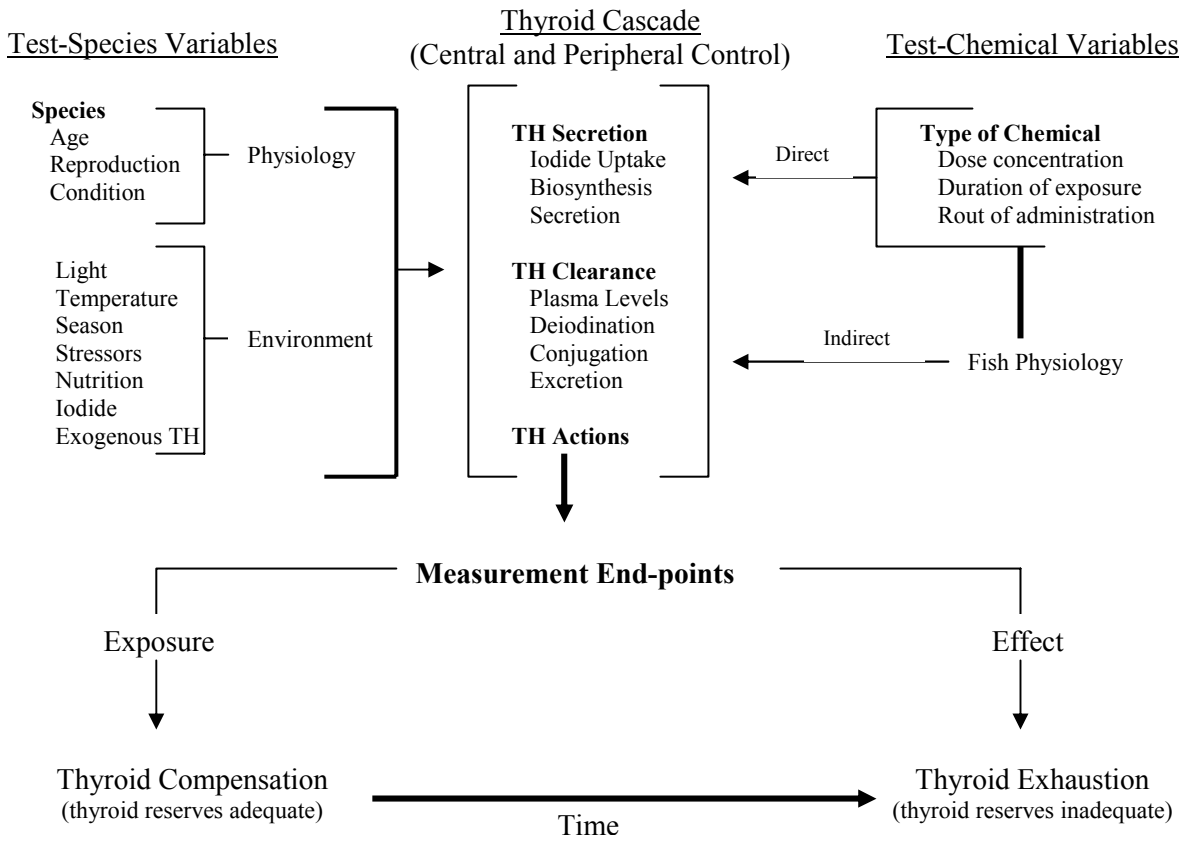
339. Metamorphosis of Japanese flounder is characterized, in part, by shortening of the dorsal fin rays. In this test of thyroid hormone action, dorsal fin rays of Japanese flounder were removed with part of the skull from the body and kept in an ice-cold Hank's solution containing 1% bovine serum albumin and 60 ug/ml kanamycin sulfate (DeJesus et al. 1990). Five fin rays were randomly distributed into each designated culture bottle containing 5 ml of culture medium supplemented with different concentrations (0.001 to 1.0 ug/ml) of T_4 or T_3. Cultures were kept at 20˚C for 7 to 8 days and the extent of shortening of the dorsal fin was monitored by measuring the length of the second fin ray. T_3 was more potent than T_4,

but both accelerated shortening of the fin rays. T_3 was effective at a concentration of 0.001 ug/ml, whereas 0.1 ug/ml of T_4 was required. The earliest statistically significant actions of T_3 were detected after 3 days, whereas T_4 actions required 8 days. This assay can also be done *in vivo.* A significant action of 0.01 ug T_4/ml seawater was detected in 5 days *in vivo.* The advantage of this assay is the dose-dependency and simplicity of quantification. The larval flatfish are quite small (smaller than a dime) and can be fairly easily maintained. The disadvantage is the paucity of breeding sites for flatfishes, although this is improving.

5.8.4 Recommended Assay Protocols

340. A recent review paper on thyroid function in fish by Brown et al. (2004) stated that "a xenobiotic-induced change in fish thyroid function has yet to be conclusively causally linked to decreased fitness of survival." The attribution of xenobiotic effects to the thyroid function in fish is exceedingly complex (Figure 5-2). One must consider numerous variables, not the least of which is distinguishing the differences between indirect and direct actions on the thyroid cascade from chemical exposure (thoroughly reviewed by Brown et al. 2004).

Figure 5-2 Factors to be considered when evaluating chemical exposure to fish and potential thyroid function impairment (TH = Thyroid hormones) (Adapted from Brown et al. 2004)



341. Currently, the centrally controlled thyroidal secretion of T_4 can be monitored adequately from the plasma total and free T_4 levels and from thyroid or thyrotrope histological appearance (Eales et al. 1999).

342. The peripherally controlled conversion of T_4 to T_3 cannot be monitored reliably from plasma T_3 levels alone. However, deiodination activities (e.g., *in vitro* assessments of a suite of rate-limiting deiodinations in the liver and brain) should adequately evaluate peripheral thyroidal (T_3) status (Eales et al.

1999). Eales et al. (1999) propose deiodination assay methods that could be used as sensitive indices of peripheral change and as biomarkers of exposure.

343. Assays specific for post-receptor biologic actions of T_3 are difficult to develop for fish. This is because T_3 acts permissively with other hormones that may be affected by a xenobiotic independently of any change in thyroidal status. Eales et al. (1999) urge that such assays be developed, however, to provide indicators of effect on both individuals and populations. One whole animal assay with promise is the proliferation of epithelial cells in the developing stomach. Immersion of summer flounder in 0.39 mM thiourea clearly disrupts proliferation, whereas addition of 13 nM thyroxine causes a 5- to 10-fold increase in proliferation within 2 days (Soffientino and Specker 2001, 2003). This mitogenic action of thyroxine is most likely to be a direct effect and not a permissive effect.

344. Based upon this literature review, there are currently no *in vitro* or *in vivo* assays that are sufficiently developed to warrant recommendation for use to efficiently screen chemicals for thyroid disruption. Methods are available that can be used to measure thyroid hormones (T_4 or T_3) in plasma and tissue, such as enzyme linked immunosorbent assay (ELISA) or radioimmunoassay (RIA) methods, deiodinase and thyroid hormone receptor assays (Bres et al. 1994; Cerda-Reverter et al. 1996). However, our ability to accurately interpret the causes and implications of potential alterations in T_4 or T_3 levels in teleosts is limited. Additional research would be required before an effective thyroid screen would be available for this piscine group. In a workshop on screening methods for thyroid hormone disruption (DeVito et al. 1999), participants failed to recommend a single fish thyroid assay, instead recommending an amphibian assay for use in nonmammalian wildlife. However, it was suggested that many of the assays put forward for mammalian systems could be appropriately adapted for use in fish, with additional research and refinement (DeVito et al. 1999). Thyroid function can also be examined histologically in fish; however, histological examinations are confounded, because the thyroid gland in most teleosts is not encapsulated and consists of diffuse scattered follicles, making such examinations difficult. However, measures of vascularization, epithelial cell height, and the T_4 colloidal ring reflect exposure to stimulation or inhibition and can be determined if enough follicles are sectioned.

345. Additional research and refinement of *in vivo* assays, such as the flatfish metamorphosis assays, could be adapted to be used in screening for thyroid function in fish. However, flatfish is not the best model candidate for laboratory testing, due to its current limited availability. Also, the *in vitro* deiodination assay shows promise with additional research and refinement as a screening tool that could be used as an effective thyroid assay in the future.

5.9 Summary

346. In most teleost fish the thyroid tissue is distributed diffusely around vascular tissue in the basibranchial region. The fish thyroid cascade can be broken down into the following three elements. First is the centrally controlled brain-pituitary-thyroid axis. The brain-pituitary-thyroid axis is primarily responsible for synthesis, storage, and secretion of T_4 and maintenance of T_4 levels for a given physiological state. The second element is the peripherally controlled (e.g., in liver tissue) availability of the active hormone T_3. The primary production of the biologically active form of the thyroid hormone T_3 is via outer-ring monodeiodination of T_4, which occurs in peripheral tissues (e.g., liver tissue). In most teleosts this occurs in peripheral organs or tissues such as the liver. The third phase is the receptor-mediated effects of T_3 on target cells to regulate development, growth, and aspects of reproduction.

347. While similarities exist between mammalian and fish thyroidal systems, one important distinction exists: in mammals, the thyroid system is principally driven by the central brain-pituitary-thyroid axis, regulating both T_4 and T_3 secretions via negative feedback by both T_4 and T_3 on the brain-pituitary thyroid axis. In contrast, the fish thyroidal system does not appear to be centrally driven (i.e., via brain-pituitary-

thyroid axis) but instead is under peripheral control. This deference has important implications regarding measurement of thyroidal status in fish. Studies have demonstrated that massive experimental increases of T_4 in fish did not increase T_3 levels, concluding that increases in T_4 do not drive T_3 production (Eales et al. 1999).

348. Upon completion of the literature review for this chapter, a sufficiently developed assay for fish thyroid function was not found. In addition, no standardized protocol/method had been validated to assess thyroid disruption in fish.

349. A series of measurement endpoints have been put forward to assess thyroid function at different levels of the fish thyroid cascade (Eales et al. 1999; Brown et al. 2004). A deiodination assay in addition to other biomarkers and morphological responses, put forward in Table 5-1 below, attempts to monitor activity throughout the thyroid cascade 1) central, 2) peripheral, and 3) receptor-mediated effects.

350. Measurement of the central control of the thyroid cascade could be accomplished via histological investigation of thyroid follicles, in addition to measurement of plasma total and free T_4 levels. T_4 levels can be measured via RIA or ELISA method.

351. The peripheral control of the conversion of T_4 to T_3 cannot be adequately assessed by the simple measurement of plasma T_3 alone (Eales et al. 1999). This is due to several reasons: 1) much of the T_3 produced by a given tissue will not enter the plasma; 2) the plasma T_3 level is not indicative of the T_3 availability to all tissues, just those that rely on T_3 from plasma, such as the kidney; and 3) T_3 levels are very well buffered against perturbations and thus are not a very sensitive measurement (large variations in T_3 levels do not cause significant changes in receptor-mediated effects on target cells) (Eales et al. 1999; Brown et al. 2004). The formation of T_3 from T_4 can be assessed via a suite of rate-limiting deiodinase activity from liver or brain tissue (Eales et al. 1999; Brown et al. 2004). Conducting a deiodination assay along with the measurement of plasma T_4 and T_3 levels should provide a good indication of the peripheral thyroid system status (Eales et al. 1999; Brown et al. 2004).

352. A recent review paper (Brown et al. 2004) failed to find a satisfactory assay for evaluation of postreceptor effects of T_3. This was true for our review of the literature as well. However, biological responses that are unique to thyroid function such as parr-smolt transformation, flounder metamorphosis, and young fish early development (metamorphosis from post hatch to fry) with additional research could become effective thyroid hormone screens/tests. Consideration should be given to size of fish test species in addition to the time required to conduct the test. Because of this, it is recommended that future research be focused on very young developing fish, an area that has not been thoroughly explored but a life stage that may prove to be very susceptible to thyroid disruption (Brown et al. 2004). Research is also recommended in the development of biochemical and molecular biological techniques geared toward very young fish (metamorphosis from post hatch to fry) (Brown et al 2004). These could be used in addition to other thyroid endpoints, such as follicular histology and T_4 and T_4 assays.

353. Table 5-2 shows generalized points of thyroid disruption in fishes. Specific points of disruption are difficult to tabulate because of the large diversity of fishes and heterogeneity in some of the key proteins mediating specific roles within the HPT axis. Points of disruption are clustered within categories, with more focused assays proposed to test for disruption within these categories.

Table 5-1 Status of Selected Assays for Thyroid System Effects in Fish

Assay Name	Species	Major Thyroid-Related Endpoints	Target Effects Relevant to the Thyroid System	Status of the Assay	
				Advantages	Disadvantages
Measurement endpoint in a Fish Thyroid Assay	Teleost	Centrally Controlled (Brain-pituitary-thyroid axis) Thyroxine (T_4) synthesis and secretion	Histological evaluation of thyrotrope and measurement of T_4 prohormone	Straightforward histological and hormone measurement endpoint	Does not consider the "Peripherally controlled Thyroid hormone or the Receptor-mediated effects of T_3 on Target cells. Relevance to other taxa, especially mammals, is unknown
Measurement endpoint in a Fish Thyroid Assay	Teleost	Peripherally Controlled -T_3 synthesis and secretion.	Deiodinase assay. Measures tissues potential to either form or degrade T_3	Deiodination activities in tissue (e.g., liver T_4ORD) in conjunction with plasma T_4 and T_3 levels provide detection of most xenobiotic effects on the thyroid peripheral system.	Measurement of thyroidal peripheral system only. Does not consider other components of the fish thyroid cascade, such as central T_4 production (Brain-pituitary-thyroid axis) It also does not consider receptor mediated effects of T_3 on target cells. Relevance to other taxa, especially mammals, is unknown
Flounder metamorphosis assay	Flounder	Metamorphosis of flounder from symmetrical to asymmetrical fish. Migration of eye, mouth and fin ray development.	Normal, delayed, or accelerated morphogenesis from larva to juvenile	Straightforward morphological and behavioral endpoints, reflecting integrated effects of thyroid hormones	Does not consider other components of the fish thyroid cascade, such as central T_4 production (Brain-pituitary-thyroid axis). Relevance to other taxa, especially mammals, is unknown. This assay requires further development and refinement, standardization and validation

Table 5-2 Points of Thyroid Disruption in Fish

Disruption or Evaluation Site	Endpoints of Interest	Target Effects of Disruption	Assay Availability	Status of Assay
Centrally controlled thyroidal secretion of T_4	Plasma T_4 and histological examination of thyroid	An indication of thyroid follicle function ability to secrete T_4 prohormone.	Yes	RIAs and ELISAs; and histological examination in common use.
Peripherally controlled conversion of T_4 to T_3	Converting enzymes (deiodinase) in a given tissue to either form or degrade T_3	Deiodination of T_4 to T_3. The ability to convert T_4 to the biologically active form T_3	Yes	Deiodination Assays
Thyroid Hormone Receptor (TR)	TR affinity and binding capacity	Receptor-mediated effects of T_3 on target cells	Yes	TRs have been cloned and Sequenced in a variety of fishes and can be used for this assay.
Post receptor mediated effects	Flounder metamorphosis (or larval fish growth and metamorphosis)	Ability of larval fish to undergo normal metamorphosis i.e., flounder or larval fish to adult).	Yes	A flounder metamorphosis assay has not been fully developed, standardized and validated to specifically detected chemicals that disrupt thyroid function.

6.0 THE HPT AXIS IN FROGS AND ITS ROLE IN FROG DEVELOPMENT AND REPRODUCTION

[Editor's note: Some material in this section was taken from a detailed review paper previously prepared for U.S. EPA on Amphibian Metamorphosis Assays. Material has also been adapted from OECD DRP No. 46, Detailed Review Paper on Amphibian Metamorphosis Assay for the Detection of Thyroid Active Substances (OECD 2004). These materials have been included here because they are considered to be especially relevant to the purposes of this DRP.]

6.1 Overview of the HPT Axis in Frogs

354. The endocrine system, also referred to as the hormone system, consists of glands and secretory cells located throughout the body, hormones that are synthesized and secreted by the glands into the bloodstream, receptors in the various target organs, and tissues that recognize and respond to the hormones. Normal function of the endocrine system, therefore, contributes to homeostasis (the body's ability to maintain itself in the presence of external and internal changes) and to the body's ability to control and regulate reproduction, development, and behavior. The function of the system is to regulate a wide range of biological processes, including control of blood sugar (through the hormone insulin from the pancreas); growth and function of reproductive systems (through the hormones testosterone and estrogen and related components from the testes and ovaries); regulation of metabolism (through the hormones cortisol from the adrenal glands and thyroxin from the thyroid gland); development of the brain and the rest of the nervous system (estrogen and thyroid hormones); and development of an organism from conception through adulthood and old age. An endocrine system is found in nearly all animals, including mammals, non-mammalian vertebrates (e.g., fish, amphibians, reptiles, and birds), and invertebrates (e.g., snails, lobsters, insects, and other species).

355. As summarized by Hayes (2000), the function of the amphibian endocrine system is reasonably consistent with vertebrate hormonal axes, with several exceptions. Anatomically, the amphibian endocrine axis displays greater similarity to mammals than teleosts. However, the adenohypophysis in amphibians is less regionalized than other tetrapods, and not separated into distinct zones as in teleosts. Similarly, the hypothalamus is less divided into nuclei than other non-mammalian tetrapods, birds, or mammals. Physiologically, corticotropin releasing factor (CRF) is the primary thyrotrope in larval amphibians, rather than thyrotropin releasing hormone (TRH) (Denver, 1993; 1997, 1998; Denver and Licht, 1989). Hayes (1997) also found that corticosterone was capable of providing negative feedback directly on the pituitary, and potentially the hypothalamus in larval amphibians. These relationships are not found in adult amphibians, as TRH acts as the primary thyrotrope. As in most vertebrate endocrine systems, tropic hormones are released from the pituitary as the result of pituitary stimulation by releasing factors secreted by the hypothalamus (Hayes 1997a). External environmental stressors and input from the central nervous system influence hypothalamic activity. It is now reasonably well understood that synthesis of TH in the thyroid is under the direction of complex neuroendocrine pathways. TH, in turn, completes a complicated feedback loop at the central nervous system (CNS) and pituitary levels. These interactions form a complex pathway referred to as the hypothalamus-pituitary-thyroid (HPT) axis (Shi 2000).

356. In terms of anatomical organization of the HPT axis, the amphibian neuroendocrine system exhibits most of the general characteristics of mammals and lacks the distinguishing structures that characterize the teleosts. A helpful description of endocrine control of amphibian metamorphosis is presented in Shi (2000). In most tetrapods, the median eminence and the pars nervosa are well-developed and have distinct neurohemal function. The amphibian pars distalis displays marginal cellular regionalization, whereas, reptiles and birds demonstrate two reasonably distinct regions. However, these zones are not divided into a rostal and proximal zone as in fishes. The pars tuberalis, which contains

secretory cells, is a consistent feature amongst most tetrapods, although a specific physiological function has not yet been defined.

6.1.1 Hypothalamus

357. Of the tetrapods, the amphibian hypothalamus is less differentiated into specific nuclei than that found in reptiles, birds, and mammals, respectively (Norris 1996). In most tetrapods, the control of the adenohypophysis is accomplished by neurovascular means rather than by direct neuronal input. Amphibians, however, appear to have some direct neural control over the pars intermedia. The preoptic area contains several specific neurosecretory centers, including the lateral, medial, and the preoptic nucleus. In amphibians, the preoptic region of the hypothalamus is responsible for synthesis of gonadotropin releasing hormone (GnRH), growth hormone releasing hormone (GHRH), somatostatin (SS), arginine vasotocin (AVT), thyrotropin releasing hormone (TRH), corticotropin releasing factor (CRF), and an oxytocin-like peptide referred to as mesotocin. The preoptic nucleus is further subdivided into the suprachiasmatic nucleus and the ventromedial nucleus in the posterior region. AVT has been found in the suprachiasmatic nucleus. The infundibular nucleus is located in the basal region of the hypothalamus and provides aminergic and peptidergic fibers to the median eminence. The infundibular nucleus is further subdivided into dorsal and ventral regions and is homologous to the primary hypophysiotropic region of the mammalian hypothalamus (Norris 1996). TRH and SS-like peptides have been located in the dorsal regions, and TRH and melanocyte stimulating hormone (MSH) in the ventral regions, of the infundibular nucleus. A pituitary adenylate cyclase activating peptide which stimulates cAMP production in the anuran pars distalis has also been found in the infundibular nucleus. The neuropeptide [a pituitary adenylate cyclase activating peptide] appears to play a role in pituitary control by the hypothalamus (Yon et al. 1993). The influence of the hypothalamus on metamorphosis is mediated through induction of the release of TSH from the pituitary. Originally, it was thought that TRH was responsible for inducing the secretion of thyroid stimulating hormone (TSH) from the pituitary in a similar pathway found in most mammals (Shi 2000). However, it is now clear that TRH does not regulate TSH during metamorphosis. TRH acquires the ability to regulate TSH following the completion of metamorphosis. Further, evidence now supports a role for CRF in the regulation of TSH during metamorphosis (Denver et al. 2002; Okada et al. 2003). Historically, the importance of the hypothalamus in the control of metamorphosis has been demonstrated by hypothalectomy, pituitary transplant to a remote part of the body, or providing an impermeable barrier between the hypothalamus and the pituitary gland in frogs (Dodd and Dodd 1976; White and Nicoll 1981; Kikuyama et al. 1993; Kaltenbach 1996, Denver 1996). High concentrations of TRH have been detected in the brain and skin of *R. pipiens* (Jackson and Reichlin 1977). Further, in *X. laevis* and *R. catesbeiana* brain tissue, TRH levels have been found to increase throughout metamorphosis and metamorphic climax (King and Miller 1981; Bray and Sicard 1982; Millar et al. 1983; Balls et al. 1985; Mimnagh et al. 1987). However, a paradoxical relationship appears to exist between TRH and the rate of metamorphosis (Shi 2000). More specifically, TRH is capable of inducing the release of TSH from the adult anuran pituitary, but not the tadpole pituitary (Denver 1988; Denver and Licht 1989; Okada et al. 2003). While TRH has generally been found to have no effect on the rate of metamorphosis, CRF is capable of accelerating it (Dodd and Dodd 1976; White and Nicoll 1981; Denver and Licht 1989; Kikuyama et al. 1993; Kaltenbach 1996; Denver 1993; 1996; 1998).

358. In mammals, CRF is responsible for inducing the secretion of corticotropin (ACTH). Further experimentation demonstrated that mammalian CRF is also capable of accelerating ACTH release from frog pituitaries (Tonon et al. 1986; Gracia-Navarro et al. 1992). Not surprisingly, ACTH does not induce the thyroid to produce thyroid hormone (TH) (Sakai et al. 1991). CRF is now shown to act directly on the amphibian pituitary gland, stimulating the release of TSH (Denver 1988; Denver and Licht 1989; and Jacobs and Kuhn 1992; Okada et al. 2003). Because CRF is capable of raising TH levels in anurans and accelerating metamorphosis, and because the use of anti-CRF antibodies or CRF receptor antagonists slows metamorphosis; CRF appears to function as the amphibian TSH releasing factor and orchestrates

regulation of the anuran pituitary (Rivier et al. 1984; Gancedo et al. 1992; Denver 1993; 1997b). Anuran CRF genes in *X. laevis* are relatively homologous to mammalian CRF (ca. 93%) (Stenzel-Poore et al. 1992; Shi 2000). Neurosecretory cells in the preoptic region develop under the control of the thyroid during metamorphosis, as does the median eminence (Etkin 1968; Verhaert et al. 1984; Olivereau et al. 1987; Gonzalez and Lederis 1988; Carr and Norris 1990; Stenzel-Poore et al. 1992). These findings generally agree with the suggestion by Denver et al. (1997) that a hypothalamic feedback loop exists at the pituitary level that connects the positive maturational actions of TH on neuroendocrine development (Carr and Norris 1990). Overall, the primary significance of this research is that CRF, not TRH, is the primary hypothalamic releasing hormone responsible for the induction of metamorphosis (Carr and Norris 1990; Denver 1996; Denver et al. 1997; Shi 2000). Further discussion of the role of TRH, TSH, CRF, and TH in amphibian metamorphosis can be found in OECD DRP No. 46, Detailed Review Paper on Amphibian Metamorphosis Assay for the Detection of Thyroid Active Substances (OECD 2004).

6.1.2 Pituitary

359. The amphibian pituitary is generally divided into the neurohypophysis and the adenohypophysis (Norris 1996). The neurohypophysis is innervated by peptidergic fibers originating from the preoptic nucleus. AVT and mesotocin are co-localized within the pars nervosa. Some evidence supports co-localization of TRH with mesotocin (Norris 1996). A set of specific neurons has been shown to travel from the dorsal hypothalamus through the median eminence to the pars nervosa. The AVT-secreting cell bodies in the preoptic area and the axonal endings in the pars nervosa have been shown to also contain GHRH. Other sets of neurons following the same pathway from the hypothalamus to the pituitary have been shown to contain MSH. The adenohypophysis contains three distinct regions including the pars tuberalis, pars intermedia, and pars distalis. Ultrastructural comparison of immunoreactive cytoplasmic granules suggest that two different cell types exist within the pars tuberalis. The pars intermedia has a poor vascular supply, but is innervated by aminergic neurons originating from aminergic nuclei of the hypothalamus. Secretion of MSH has been shown to be under the direct control of the aminergic, and possibly peptidergic, neurons. Neuropeptide Y (NPY) and atrial natriuretic peptide (ANP)-reactive neurons also innervate the pars intermedia of most anurans. NPY and ANP have been shown to inhibit and stimulate MSH release, respectively. The pars distalis is generally not highly regionalized, although some localization based on cellular subtypes exists, particularly in urodeles. Much of the focus of studies of amphibian pituitary function has been on the activity of the pars distalis. Extensive research focus has been placed on the pars distalis since it is considered the primary endocrine region of the pituitary and source of tropic hormones (Norris 1996). TSH, gonadotropic hormone (GTH), ACTH, PRL, and GH are produced and released from the pars distalis.

6.1.3 Tropic Hormones

360. As in mammals, tropic hormones in amphibians, as well as most vertebrates, are generally categorized as the gonadotropins (leutinizing hormone [LH] and follicle stimulating hormone [FSH]), prolactin [PRL], and TSH, growth hormone (GH), MSH, ACTH, and other corticotropin-like substances.

361. The fundamental difference between hypothalamic control over thyrotrope production (TSH) and release from the pituitary in mammals and amphibians is that thyrotropin releasing hormone (TRH) does not appear to mediate this process in amphibians. Rather, release of TSH from the pituitary, and ultimately TH from the thyroid, is controlled by CRF. TH also provides negative feedback at the pituitary level (Denver 1993; Denver 1997a; Denver 1998; Denver and Licht 1989; and Ganecedo et al. 1992).

362. Various inter-relationships between glucocorticoids, gonadal steroids, and the thyroid axis have been found to occur in developing amphibians (Roth 1948; Frieden and Naile 1955; Jaffe 1981; Kobayashi 1958; Kikuyama et al. 1983; Krug et al. 1983; Leatherland 1985; Galton 1990; Gray and Janssens 1990;

Leloup-Hatey et al. 1990; Hayes et al. 1993; Kikuyama et al. 1993; Hayes 1995a; Hayes 1995b, Hayes 1997b, and Hayes 2000). These endocrine pathway interactions are described in more detail in the following sections. In summary, TH interactions with glucocorticoids include: 1) TH-induced production of corticoids by the interrenal gland, and 2) increased titers of T_3 via conversion from T_4 that are induced by corticoids. Both processes increase the activity of the thyroid axis. In contrast, sex steroids repress the activity of the thyroid axis directly opposite to the effect of the corticoids. TH interaction with gonadal steroid hormones include: 1) inhibition of T_4 to T_3 conversion, 2) establishment of a negative feedback mechanism at the pituitary level, ultimately slowing the production and secretion of TH. In addition, numerous hormone interactions with the thyroid axis may occur at the receptor level, including: 1) corticoid enhancement of TH activity by increasing TH binding sites (i.e. TR expression) (Niki et al. 1981; Suzuki and Kikuyama 1983), 2) TH facilitation of steroid receptor induction in anurans (Hayes 1997b), and 3) induction of TR synthesis by T_3 (Rabelo and Tata 1993; Rabelo et al. 1994; Tata 1994; Ulisse and Tata 1994).

6.1.4 Significance of the HPT Axis as a Target for EDCs (portions excerpted from OECD DRP No. 46, Detailed Review Paper on Amphibian Metamorphosis Assay for the Detection of Thyroid Active Substances (OECD 2004))

363. Anthropogenic compounds, as well as naturally occurring chemicals, have the potential to disrupt the endocrine system of animals, including humans (Colborn and Clement 1992). Among the anthropogenic contaminants suspected to interfere with vertebrate and invertebrate endocrine systems are the persistent, bioaccumulative organic compounds including pesticides, industrial chemicals, as well as some metals (Brucker-Davis 1998). It is suspected that wildlife populations are already adversely affected by these compounds. Lister and Van der Kraak (2002) and McMaster et al. (2001) summarized the potential impacts of EDCs in various wildlife, including but not limited to: 1) thyroid dysfunction in birds, amphibians, and fish; 2) decreased fertility in birds, amphibians, fish, shellfish, and mammals; 3) decreased hatching success in birds, fish, alligators, and turtles; 4) gross birth defects in birds, amphibians, fish, and turtles; 5) metabolic abnormalities in birds, fish, and mammals; 6) behavioral abnormalities in birds; 7) demasculinization and feminization of male fish, amphibians, birds, and mammals; 8) defeminization and masculinization of female fish, amphibians, alligators, and birds; 9) and compromised immune system in birds and mammals.

364. The term “endocrine disruption” and the hypothesis that such agents exist in the environment that affect reproduction and development dates back to the late 1980s (Colborn and Clement 1992; Kavlock et al. 1996). These authors described such effects in fish-eating birds, alligators, Great Lakes mink, frogs, invertebrates, and humans. They suggested that these chemicals served as agonists or antagonists to endogenous endocrine hormonal axes to disrupt the hormonal control of homeostasis, cellular differentiation, embryonic growth, and development, and notably included effects on reproductive organs and reproductive function. These agents were called endocrine-active chemicals (EACs), endocrine-disrupting chemicals (EDCs), or most popularly “endocrine disruptors” (EDs) (EDSTAC 1998).

365. Reduced growth, reproductive dysfunction, abnormal behavior, and abnormal development from exposure to a variety of natural and anthropogenic chemicals in invertebrates, fish, amphibian, reptilian, avian, and mammalian species have been recently demonstrated (Lister and Van der Kraak 2002; McMaster et al. 2001). Although EDCs are now thought to adversely affect development, reproduction, and general homeostasis in a wide variety of different taxa, several other issues complicate the evaluation of EDCs in vertebrate animals: 1) the chemicals of concern may have entirely different effects on the embryo, fetus, or perinatal organism than on the adult; 2) the effects are most often manifested in offspring, not in the exposed parent; 3) the timing of exposure in the developing organism is crucial in determining its character and future potential; and 4) although critical exposure occurs during embryonic development, obvious manifestations might not occur until maturity (Kavlock et al. 1996). It is also

possible to have differing effects of the same compound in different species or tissues, presumably due to differences in receptors.

366. It should be noted that metamorphosis and, in some cases, thyroid function can be influenced by a combination of other biotic and abiotic factors beyond the realm of chemical stressors (Stearns 1989). These factors include temperature, water availability, crowding, light, diet, and environmental iodine levels (Dodd and Dodd 1976). Amphibian larvae respond to changes in these factors through high levels of plasticity in the phenotypes (Denver 1997a; Denver 1998). Some factors that inhibit growth when present during premetamorphic stages are also capable of inducing rapid metamorphosis when present during prometamorphosis. These factors include crowding, resource limitation, habitat desiccation, and predation (Denver 1997a; Denver 1998). Temperature also affects the rate of metamorphosis such that greater temperatures stimulate the rate of metamorphosis (Hayes et al. 1993), whereas lower temperatures slow down TH-induced metamorphosis (Dodd and Dodd 1976). The effects of temperature may be due to reduction in TH binding at the tissue level, changes in neuroendocrine control of TH synthesis, or more generalized effects on metabolism (i.e., Q10 effects on enzyme kinetics) (Tata 1972; Dodd and Dodd 1976). Overall, it must be understood that the link between the thyroid axis and metamorphosis can be influenced by different external factors and/or combinations of external factors. This phenomenon is not unique to the thyroid system, and external factors are capable of affecting other developmental processes.

6.2 Hormone Synthesis

6.2.1 Anatomy of the Amphibian Thyroid

367. Generally, the vertebrate thyroid is a highly conserved structure. In amphibians, the thyroid glands exist as a paired set of masses of highly vascularized follicles encased by a connective tissue capsule. Follicular structure and function are highly mammalian-like.

6.2.2 Development of the Amphibian Thyroid

368. To facilitate the description of the morphological development of the thyroid, a comparison between *Xenopus* development and development in *Rana* is provided in Table 6-1. The thyroid gland in most amphibians develops during late embryogenesis (Dodd and Dodd 1976; Regard 1978).

369. In *X. laevis*, the thyroid develops from a pharyngeal epithelial ridge around NF stage 35 (Nieuwkoop and Faber 1994). Following division of the thyroid, follicular development is first present by NF stage 44. A functional thyroid gland with numerous follicles is present by NF stage 53. Follicular development continues resulting in growth of the gland throughout prometamorphosis. Concurrently, TH synthesis and secretion into the circulatory system increases in preparation for metamorphosis and peaks with a surge at the onset of metamorphic climax. After metamorphosis is complete, the thyroid gland continues to grow until it reaches adult size although reduced activity compared to metamorphosis is noted. During this time the glandular epithelium shifts from a columnar to a cuboidal morphology which is characteristic of reduced TH production. Two naturally occurring THs: 1) 3,5,3',5'-tetraiodothyronine (T_4 or thyroxine), and 2) 3,5,3'-triiodothyronine (T_3) have been found in anuran species. Based on nearly 100 years of research, the effect of TH on amphibian metamorphosis is no longer debated, although research in understanding the functional mechanisms and interaction with other hormonal pathways continues today (Gudernatsch 1912; Allen 1916; Allen 1929; White and Nichol 1981; Tata 1968; Dodd and Dodd 1976; Brown et al. 1995; Shi 2000).

6.2.3 Regulation of TH Synthesis by TSH

370. The pituitary hormone thyrotropin (or thyroid stimulating hormone [TSH]), produced and secreted by the pars distalis region of the pituitary gland, is primarily responsible for inducing the

production and release of TH from the thyroid gland (Shi 2000). TSH production and release is controlled via negative feedback at the pituitary level (Dodd and Dodd 1976; White and Nicoll 1981; Kikuyama et al. 1993; Kaltenbach 1996; Denver 1996). Until recently, traditional measures of plasma TSH have not been successful in amphibians due to a lack of an appropriately developed assay in amphibians. Okada et al. (2003) have recentely developed a homologous radioimmunoassay (RIA) for bullfrog TSH. Sakai et al. (1991) found that both purified frog and purified bovine TSH stimulated the release of T_4 from the thyroid gland. In hypophysectomized *X. laevis* tadpoles, Dodd and Dodd (1976) estimated TSH levels in crude pituitary extracts during development using radioiodine uptake. This work suggested that TSH was detectable at trace levels during prometamorphosis (Nieuwkoop and Faber [NF] stage 56), but increased markedly at the onset of metamorphic climax (NF stage 59). In these studies, a decrease in pituitary TSH levels at stage 61 followed by a spike in pituitary TSH at stage 62 was found. Thus, increasing levels of TSH occur during metamorphosis when TH is required. Coincidently, TSH production and secretion rises and is high at metamorphic climax when the plasma TH levels are at their greatest. The biosynthesis and secretion of TSH drives the increase in plasma TH. Negative feedback by TH on TSH synthesis returns TSH production to a level in the adult that is comparable to that of the early prometamorphic larvae. An understanding of this process at the molecular level has been achieved as the result of the production of complementary DNAs (cDNAs) coding for TSH in *X. laevis* (Buckbinder and Brown 1993). Buckbinder and Brown (1993) essentially found that messenger RNA (mRNA) levels during metamorphosis indicated that TSH genes were activated around NF stage 53, immediately prior to the first stage in which pituitary TSH levels are detectable. TSH levels peak at approximately NF stages 58 or 59, and drop to appreciably lower levels toward the conclusion of metamorphosis (Dodd and Dodd 1976; Shi 2000). Manzon and Denver (2004) have shown recently through the use of semi-quantitative RT-RCR that TSH expression does not decrease until the end of metamorphic climax. TSH gene repression subsequent to stage 59 coincides with high levels of plasma TH. This finding is consistent with a TH-induced negative feedback loop at the pituitary or hypothalamic levels.

Table 6-1 Comparative Larval Anuran Stages [excerpted from OECD DRP No. 46, Detailed Review Paper on Amphibian Metamorphosis Assay for the Detection of Thyroid Active Substances (2004), with modification]

Specie Stages			Morphological	Metamorphic
X. laevis[5]	***R. pipiens***[6]	**Anuran**[7]	**Landmarks**	**Event**[8]
46	I	26	Limb Bud Growth	Premetamorphosis
47/48	II	27		
49/50	III	28		
51	IV	29		
52	V	30		
53	VI	31		
53	VII	32		
53	VIII	33		
54	IX	34	Digit Differentiation	Prometamorphosis
55	X	35		
55	XI	36		
55	XII	37		
56	XIII	38		
57	XIV	39		
57	XV	40		
58	XVI	40	Rapid Hind Limb Growth	
59	XVII	40		
60	XVIII	41		
60	XIX	41		
61	XX	41		
62	XXI	42	Rapid tail resorption begins	Climax
63	XXII	43		
64	XXIII	44		
65	XXIV	45		
66	XXV	46		

[5] Nieuwkoop and Faber (1994)

[6] Taylor and Kollros (1946)

[7] Gosner (1960)

[8] Dodd and Dodd (1976)

6.2.3.1 TSH Biochemistry

371. Interestingly, Buckbinder and Brown (1993) and Kikuyama et al. (1993) found a relatively high degree of homology between anuran TSH cDNA and mammalian species. The degree of homology is approximately 60 to 70 percent at the amino acid sequence level, according to the works cited. In amphibians, TSH is a glycoprotein comprised of two polypeptide subunits (α and β). The α subunit is consistent structurally with other proteinaceous hormones including FSH and LH, whereas the β subunit confers specificity to TSH (Pierce and Parsons 1981; Kaltenbach 1996; Denver 1996).

6.2.3.2 TSH Genes

372. TSH genes have been cloned (cDNAs) in *X. laevis* (Buckbinder and Brown 1993) encoding for both subunits and used as a diagnostic tool to measure the time course of expression through metamorphosis. Buckbinder and Brown (1993) found that expression of both subunits was in parallel and occurred between NF stages 54 and the conclusion of metamorphic climax with a peak expression around NF stages 58/59. A single mRNA species of ca. 700 bases was detected for TSHα, whereas TSHβ consisted of three hybridizing species of 4.4, 2.4, and 0.7 kb expressed in similar abundances. Further work identified that the two larger TSHβ were extensions at the 3'end and that each of the three mRNAs differed only in the poly(A) site. Greenspan (1997) and Collingwood et al. (2001) have shown that in contrast to the majority of TR-regulated genes in which up-regulation of promotor activity is controlled by TH, the TSHα promotor is regulated by a negative feedback loop in which the unliganded TR activates expression and the addition of TH results in repression. Collingwood et al. (2001) further demonstrated that regulation of TSHα expression was mediated through control of its promotor via chromatin remodeling induced by TR.

6.2.3.3 TSH Receptor and Signal Transduction

373. The polypeptide associated with this transcript consisted of a 398-amino acid residue (amino terminus) constituting a putative extracellular domain connected to a 346 amino acid residue domain on the carboxy terminus that contained a series of transmembrane segments (Parmentier et al. 1989). Expression of the cDNA conferred TSH-responsiveness in the *Xenopu*s oocytes, Y1 cells, and a TSH-binding phenotype to COS cells. These studies demonstrated that the TSH receptor (as well as the LH receptor) constitutes a sub-family of G protein-coupled receptors with distinct sequence characteristics. Thus, signal transduction proceeds via a G-protein mediated messenger cascade.

6.2.4 Thyroglobulin Synthesis

374. Essentially thyroglobulin and iodoprotein synthesis is similar in all vertebrates (Norris 1996). As in mammals, thyroglobulin synthesis occurs in the rough endoplasmic reticulum and is packaged into secretory granules by the Golgi apparatus. The synthesis of TH occurs in follicular cells and involves the synthesis of thyroglobulin and the substitution of inorganic iodide to the tyrosine residues. The final step links two iodinated tyrosine residues contained within the thyroglobulin molecule to form the iodinated TH.

6.2.5 Regulation of Iodine Uptake

375. The primary source of iodide in amphibians is dietary and absorbtion from the water supply. Inorganic iodide is absorbed from the gastrointestinal tract or the gills in larval amphibians into the circulatory system. As in mammals, the follicular cells of the thyroid gland selectively accumulate iodide (Norris 1996). Iodide is co-transported with Na^+ at the basal membrane and passively diffuses across the apical membrane into the colloid. Translocation of inorganic iodide from the apical surface and conversion to organic forms, such as the iodinated tyrosines, enhance the iodide uptake process. The

follicular iodide transport process is dependent upon an ATPase-related mechanism and is not affected by other halide anions. More specifically, the Na+/I- symporter (NIS) is reported to be an intrinsic membrane protein (618 amino acids and 65.2 kDa) with 12 putative membrane domains (Levy et al. 1997). These investigators further identified a direct correlation between circulating levels of TSH and NIS expression *in vivo* in rats. Ultimately, this process effectively concentrates iodide in the follicular cells relative to the plasma. It is anticipated that similar processes exist in metamorphosing amphibians (Norris 1996). The oocytes of oviparous vertebrate animals, including some amphibians, readily accumulate large amounts of iodide. This process ensures that the developing larva has adequate iodide required for the synthesis of TH until adequate dietary sources are available. Although iodide is accumulated throughout most of larval development, the release of TH is not necessarily related to the uptake of iodide since uptake, binding, and release of TH are independent events controlled by a variety of different factors.

6.2.6 Mechanism of Iodine Organification

376. Organification of iodide is initiated with the conversion of inorganic iodide to an active iodide which can be incorporated into the phenolic ring of tyrosine. As in mammals, the exact chemical structure of active iodide is unknown. Active iodine is apparently formed in the colloid compartment by peroxidases located on the extracellular side of the apical membrane of follicular cells which produce hydrogen peroxides. Peroxides react with iodide to form active iodide which reacts with tyrosine residues of thyroglobulin. The binding of one iodine atom to tyrosine at the 3 position produces 3-monoiodotyrosine (MIT). A second iodine atom may attach at the 5 position of the same tyrosine molecule giving rise to 3,5-diiodotyrosine, or DIT.

6.2.7 Thyroglobulin Storage

377. The specific mechanism by which THs are formed from the iodinated tyrosine residues in unknown. The coupling process in which two DIT molecules, or one DIT and one MIT molecule, results in the formation of T_4 or T_3. This coupling process follows hydrolysis of selected peptide bonds to release small peptide fragments of 15-20 kDa from thyroglobulin. The combination of the adjacent residues in the folded, globular thyroglobulin molecular fragment, and the peptide fragments result in the coupling process.

6.2.8 Interdependency of Synthetic Events

378. Although iodide uptake, iodothyronine synthesis, and TH release are controlled by TSH, the activities are not directly linked to one another, as previously indicated. TSH independently stimulates engulfment of colloid by the follicular cells and intracellular hydrolysis to MIT, DIT, and THs.

6.3 Thyroid Hormone Release

379. As in mammals, TH release is the ultimate result of hydrolysis of thyroglobulins (Norris 1996). Engulfed colloid droplets acquired through endocytosis migrate from the apical portion of the follicular cells toward the basal region and adjoin regional lysosomes. Fusions of the colloid droplets with the lysosomes form endolysosomes which catalyze hydrolysis of the thyroglobulins. Ultimately, the endolysosomes become increasingly degranulated and this results in the release of the hydrolysis products into the cytosol. Although amino acids, MIT, DIT, and THs are potentially released during the hydrolysis, only T_3 and T_4 effectively diffuse from the follicular cell into the surrounding capillary network as cytoplasmic deiodinases catalyze conversion of MIT and DIT to tyrosine and iodide. Iodide released from thyroid deiodinated T_4, MIT, and DIT forms a pool of second or waste iodide in the follicular cell that can then be used in the iodination of newly produced thyroglobulin. This process provides a means of conserving iodide in the thyroid.

380. The primary active THs, T_4 and T_3, are synthesized directly in the thyroid gland. Metabolic conversion of T_4 to T_3, however can occur in other tissues (Fox 1983; Dodd and Dodd 1976). TH synthesis is initiated by up-regulation of the thyroglobulin gene in the thyroid, which consequently produces thyroglobulin, the precursor of T_4. An intricate set of post-translational modifications, including iodination and condensation of the tyrosine residue to produce T_4, is then required. T_4 can either be secreted into the plasma from the thyroid gland, or directly converted to T_3 in the thyroid by 5'-deiodinase. Both T_4 and T_3 can be selectively inactivated by 5-deiodinases by converting TH to either T_2 or reverse T_3, respectively. This allows different tissues to possess different ratios of T_3 to T_4 depending on their specific requirements. St. Germain and Galton (1997) located two different 5-deiodinases in anurans having different enzymatic properties and tissue distributions. Different deiodinases have been isolated and cloned in *R. catesbeiana* (Davey et al. 1995; Becker et al. 1995) and *X. laevis* (St. Germain 1994). Each different isoform was found to have distinctly different regulation patterns in different tissues, thus supporting the hypothesis of TH level regulation at the tissue level.

6.4 Regulation of Serum Thyroid Hormone

6.4.1 Measures of Thyroid Hormone and Their Interpretation

381. Measurement of TH, specifically T_4 and T_3, produced by the thyroid provides a valuable measure of thyroid status during metamorphosis. Both T_4 and T_3 can be measured using conventional serum or tissue RIAs. Both serum and tissue (whole brains, thyroid, or carcass following cardiac puncture to obtain serum) should be considered. Three different methodologies of TH analysis are currently being evaluated, 1) high sensitivity RIA, 2) ELISA, and 3) liquid chromatography/gas chromatography with mass selective detection (LC/GC-MS). The former two techniques are reasonably well established in mammals (Ekins 1999; Baiser et al. 2000), and to a lesser extent in amphibians (Galton et al. 1991). The chromatographic technique is not as well established (Moller et al. 1983; De Brabandere et al. 1998), but has significant promise, because it may be able to simultaneously analyze monoiodotyrosine (MIT), diiodotyrosine (DIT), rT_3, T_3, and T_4. In some cases it will be important to measure the concentration of free T_4 and T_3 in relation to the transport protein-bound TH, since the majority of TH is protein bound (Baiser et al. 2000). Simon et al. (2002) has recently described a new approach for the analysis of iodinated organic species in serum and whole body tissue homogenates using liquid chromatography-inductively coupled plasma-mass spectrometry (LC-ICP-MS). This method enabled the simultaneous quantification of iodide, T_4, T_3, rT_3, MIT, DIT, as well as, five additional presently unidentified iodinated molecules in *Xenopus* larvae. Overall, TH analysis will be an important component of the Amphibian Metamorphosis Assays. However, the most beneficial use of TH analysis will be in combination with the histological, morphological, and molecular test methods used. It is possible, but unlikely, that TH analysis alone will provide sufficient information to be a stand-alone measure of thyroid dysfunction.

6.4.1.1 Total Thyroglobulin

382. Suzuki and Fujikura (1994) used a double antibody RIA method to measure total thyroglobulin in tadpoles and adult *R. catesbeiana*. These investigators found that levels of serum thyroglobulin increase during pre- and prometamorphosis and peak at nearly 480 ng/mL during the onset of metamorphic climax. Thyroglobulin levels decreased slowly toward the end of metamorphosis with the lowest levels recorded in juvenile animals (ca. 150 ng/mL). Adult serum thyroglobulin levels typically range between 250 and 275 ng/mL. Overall, these levels are generally greater than those found in mammalian species and birds.

6.4.1.2 Total T_4 and T_3

383. Most TH analysis in amphibians is based on total levels in plasma or whole tissue using RIA analysis which is reasonably reliable and sensitive. Detection limits for T_4 and T_3 are typically <50 ng/100

mL and <5 ng/100 mL, respectively. In anurans (*Rana pipiens*, *R. catesbeiana, Bufo marinus, Spea hammondii, and Xenopus laevis*), a gradual rise in both T_4 and T_3 occurs during metamorphosis with a spike occurring in both at the onset of metamorphic climax. Peak levels are measured near the midpoint of metamorphic climax. During this time, circulating T_3 levels range from 75-100 ng/100 mL which is 15- to 20-times the levels measured during premetamorphosis, whereas T_4 levels range from 0.4-0.5 µg/100 mL which is ca. 7- to 10-fold greater than levels recorded during premetamorphosis. These levels both decrease sharply immediately following the conclusion of metamorphic climax. Although differences in baseline levels were reported, similar levels of T_4 and T_3 were detected by Valamparampil and Oommen (1997) in the tropical anuran, *R. curtipes*.

6.4.1.3 Free T_4 and T_3

384. Free levels of plasma T_4 and T_3 followed the same general pattern as the total TH at the various stages examined (Regard et al. 1978). Galton (1980) found that <1% of total serum T_3 and T_4 and 0.5% of the total T_3 and T_4 in the cytosolic fraction were in the free form. In either case, both the free and total TH levels in adult anurans are remarkably low compared to levels measured during metamorphosis.

6.4.2 Thyroxin Binding Proteins in Amphibians

385. In the serum, TH immediately encounters serum binding proteins, the most notable of which is transthyretin (Yamauchi et al. 1993), which transport TH to the target tissues where TH enters the cytosol (Jorgensen 1978; Barsano and DeGroot 1983; Galton 1983; Benvenga and Robbins 1993). Cellular uptake mechanisms are not well understood. T_3 and T_4 are relatively hydrophobic at physiological pH (Shi 2000). Thus, passive diffusion through the cell membrane is a possible route. However, some evidence suggests that a carrier-mediated transport process involving translocation of both the TH transporter and TH is possible (Blondeau et al. 1988; Oppenheimer et al. 1987; Robbins 1992; Ribeiro et al. 1996; Benvenga and Robbins 1993). Within the cytoplasm, TH interacts with a separate group of multifunctional proteins, collectively referred to as CTHBP (cytoplasmic TH binding proteins) (Cheng 1991). It is presently unclear whether the TH-CTHBP binding can alter cellular membrane activity, or whether it only provides a means of transport to the TR. TH transporters might play a role in transcriptional regulation by TRs (Ritchie et al. 2003).

386. Although four plasma TH binding proteins that have been identified in amphibians, TBG is not found in non-mammalian species (Shi 2000). Although most of the cytosolic transport proteins are multifunctional (i.e., aldehyde dehydrogenase), the plasma binding proteins, including TTR, are reasonably specific and at least TTR plays a major role in the TH transport process. Although it was originally thought that the role of TH protein binding in the plasma was to prevent the loss of the highly lipophylic hormones, the circulating concentrations of bound TH are markedly less than the solubility of the THs suggesting that this is not the case (Schreiber and Richardson 1997). In mammals, TBG, transthyretin, and albumin represent the primary THBP each with a greater affinity for T_4 than T_3. The contrary is true of amphibians in which TTR primarily binds T_3. Small eutharians, some marsupials, and birds utilize albumin and TTR as the primary TH distribution proteins (Richardson, et al. 1994). Albumin is the primary TH transporter in reptiles. In amphibians, however, TTR plays the greatest role in TH transport. Further, in lower vertebrates, including amphibians, these TH binding proteins have a greater affinity for T_3 than T_4 which may serve as an evolutionary adaptation (Chang et al. 1999).

387. In mammals, TTR is synthesized in the choroid plexus, but in amphibians and reptiles, it is produced in the liver (Yamauchi et al. 1993 and 1998). Compared to TTRs from other vertebrate species, bullfrog TTR is highly conserved at the TH binding sites and other important structural regions of the subunits. Yamauchi et al. (1998) found bullfrog TTR in the liver, but not the choroid plexus of metamorphosing larvae (Achen et al. 1992). From an evolutionary standpoint, the synthesis of TTR in the

metamorphosing tadpole liver preceded that of the choroid plexus in reptiles, birds and mammals. As with bullfrog TTR, TTR in *X. laevis* was found to be reasonably homologous with other vertebrate TTRs (Prapunpoj et al. 2000). Further, TTR expression was found in the liver of metamorphosing larvae, but not in the brain, or in adults. Prapunpoj and co-workers (2000) evaluated the structure and binding characteristics of recombinant xTTR. Structural difference in the regions of the TTR genes coding for the amino terminal sections of the polypetide chains of TTR have incorporated step-wise shifts of mRNA splicing sites between exon 1 and 2, ultimately resulting in a shorter and more hydrophilic amino terminus. This more primitive structure may account for preferential binding of T_3 over T_4 to xTTR.

6.5 Thyroid Hormone Transport into Tissues

6.5.1 Kinetics of Thyroid Hormone Uptake

388. Galton et al. (1986) evaluated the kinetics of TH uptake in red blood and thymus cells in larval amphibians. Both cell types contained 3-5 times greater levels of T_3 than T_4. These investigators found that the uptake of T_3, but not T_4, was facilitated by a carrier-mediated process, although not necessarily by an active transport mechanism. More recently, Friesema et al. (1999) evaluated the potential of the Na+/taurocholate (NAT) co-transporting peptide and the organic anion transporting peptide in the hepatic uptake of T_4, T_3, rT_3, and T_2 in *X. laevis*. Both uptake processes were found to participate in TH and TH precursor/metabolite uptake in the liver. Other TH transporters including analogues to the rat fatty acid translocase (rFAT) (van de Putten et al. 2003) and monocarboylate transporter 8 (MCT 8) (Friesma et al. 2003) may also play a role in TH tissue transport in amphibians.

389. Recent findings point to an important role for amino acid permeases in the uptake of THs by cells (Denver, personal communication; Ritchie et al. 1999). The T_3-inducible gene IU12 from X. laevis intestine (Shi and Brown 1993; Liang et al. 1997) encodes a subunit of a heterodimeric amino acid permease complex (Torrents et al. 1998). Recent findings by Ritchie and colleagues (1999) show that this permease complex efficiently transports T_3 and T_4 when expressed in the Xenopus oocyte expression system, but is inhibited by reverse T_3. The fact that the IU12 is a T_3-inducible gene suggests that it might play a role in mediating T_3 uptake by cells during tadpole metamorphosis (Liang et al. 1997). Other TH transporters that have been identified include organic anion transporters such as Ntcp and oatp1-3 (Abe et al. 1998; Friesema et al. 1999). The possibility of specific receptors for TTR also has been demonstrated, although this means of hormone uptake requires further investigation (Divino and Schussler 1990; Schussler 2000).

6.5.2 Cloning of T_4 and T_3 Transporters

390. Ritchie et al. (2003) provide a current review of T_4 and T_3 transporters in amphibians. Each of the transporters described in the preceding section, including the NAT, rFAT, and MCT 8 have been cloned from mammals in *Xenopus*.

6.6 Conversion of T_4 to T_3

6.6.1 Overview of Deiodinases in Amphibians

391. Two deiodinase subtypes are present in most anurans. One isoform, type II (D2), catalyzes the conversion of T_4 to T_3 in the thyroid and various target tissues, whereas the other isoform, type III (D3), selectively inactivates T_3 and T_4 by converting them to T_2 and reverse T_3 (rT_3) by removing an iodide atom from the inner ring of the hormone (Huang et al. 1999). It is thought that type III deiodinase in anurans (*X. laevis*) is responsible for protecting the tissues from circulating TH. Among the numerous publications on deiodinase assays, the most commonly cited is that of Koopdonk-Kool et al. (1993), who developed a method for measuring deiodinase activity by measuring the conversion of [^{125}I]T_3 to T_2. In most cases

deiodinase activity is not considered in evaluating thyroid function; however, differences in tissue levels of T_4 and T_3 can in some cases be explained by differing deiodinase activities. Further work will be required to fully determine the usefulness of deiodinase measurement in evaluating thyroid dysfunction.

6.6.2 Expression and Regulation of D2

392. Huang et al. (2001) suggested that the orchestration of metamorphosis and the initiation of the negative feedback loop between the thyroid gland and the pituitary are controlled by D2. These investigators suggested that the control of TH-induced changes at the tissue level is based on the extent to which variable local levels of deiodinase in one tissue can influence the T_3 concentration of another tissue. This theory assumes that one tissue is producing T_3 and another is responding to the T_3. Since generation of T_3 and its action on gene expression have been shown to be autonomous (Manzon and Denver 2004), the hypothesis offered by Huang and co-workers (2001) may be incorrect. Huang et al. (2001) suggested that if the generation of T_3 from T_4 which is catalyzed by D2 is provided for local use only, then D2 could play a significant role in the sequential timing of metamorphic change. Limb buds and tails express D2 activity early and later in metamorphosis, respectively, corresponding with the time these tissues undergo metamorphic alteration. At the climax of metamorphosis, D2 expression is activated in the anterior pituitary cells responsible for the production of thyrotropin, but not in the cells that produce proopiomelanocortin (Huang et al. 2001). Physiological concentrations of T_3, but not T_4, are capable of repressing the expression of TSH subunit β. The timing and specificity of D2 expression in the thyrotrophs of the anterior pituitary, in addition to the need for locally synthesized T_3 (tissue autonomy), suggest that D2 orchestrates the negative feedback loop at the climax of metamorphosis.

393. Manzon and Denver (2004) provide a more current and different perspective on how metamorphic timing is achieved. While D2 may play a role in the generation of T_3 within the pituitary, it most certainly does not by itself regulate the timing of metamorphosis, nor the onset of negative feedback. Negative feedback is functional well before maximal D2 levels are reached.

6.6.3 Expression and Regulation of D3

394. Like D2, D3 also provides a means of mediating hormone activities associated with metamorphosis. However, in the case of D3, Shintani et al. (2002) have suggested that tissue- specific regulation of D3 gene expression is capable of mediating the effects of PRL and GH on metamorphosis in *Xenopus*. PRL and GH both act as anti-metamorphic hormones. Further study has suggested that both hormones are also capable of inducing expression of D3 which in turn inactivates THs. On the contrary, both PRL and GH have been shown to down regulate D3 expression in the liver of *Xenopus*. Using whole cultured *Xenopus* tadpole tails, Shintani et al. (2002) demonstrated that the D3 inhibitor iopanoic acid reversed the inhibition of tail resorption by PRL in the presence of T_3. High concentrations of PRL receptor and D3 mRNA were found in the cultured tails. Combined with the previous data, these results suggest that actions of PRL on metamorphic events are at least partially mediated by tissue-specific expression of D3 mRNA.

6.6.4 Role of Deiodinases in Controlling Tissue Sensitivity to Thyroid Hormone

395. As previously indicated, D2 appears to play a significant role in establishing tissue sensitivity to TH (T_3) through a process of local hormone production and tissue autonomy. D3 is also capable of regulating metamorphic events, and could potentially do so by responding to induction by antimetamorphic hormones (PRL and GH). However, it should be noted that D3 is regulated in a developmental- and TH-dependent manner, and some scientists suggest that this may not be dependent on either GH or PRL.

6.7 Thyroid Hormone Action

396. The action of TH during development is regulated at many different levels, due in part to the presence of numerous TH binding proteins in the plasma, cytosol, and nucleus. Since many of the proteins with which TH interacts are cytosolic and many of the effects occur at a non-genomic level, it was originally thought that TH acted through cytosolic actions (Davis and Davis 1996). However, today more evidence exists that TH acts at the nuclear level mediating gene regulation via nuclear-based TR (Tata and Widnell 1966; Tata 1967; Oppenheimer 1979). TH secreted from the thyroid is carried in the plasma to various tissues by various serum TH binding proteins. At least nine cytosolic and plasma proteins are known to transport TH, although several are more significant factors (Shi 2000). The pathway and interactions of THs are effectively illustrated in Shi (2000).

6.7.1 Overview of Thyroid Hormone Receptors (TRs) in Amphibians

397. Diploid vertebrate animals, including *X. (Silurana) Tropicalis*, possess two TR genes (TRα and TRβ) (Lazar 1993). *X. laevis*, which is allotetraploid, possess four TR genes, two TRα and two TRβ (Brooks et al. 1989; Yaoita *et al.* 1990). Alternative splicing of the TRβ transcripts gives rise to two different isoforms in higher vertebrates and four different isoforms in *X. laevis* (Brooks et al. 1989; Yaoita et al. 1990). TRs belong to the superfamily of nuclear hormone receptors, including glucocorticoid, estrogen, vitamin D, and retinoic acid receptors (Evans 1988; Tsai and O'Malley 1994; Yen and Chin 1994).

6.7.2 Expression and Regulation of Alpha and Beta TRs

398. TRα is expressed in the early *X. laevis* larvae prior to the development of the thyroid gland (Yaoita and Brown 1990; Banker et al. 1991). TRα has been suggested to play a significant role in the repression of T_3 response genes prior to the onset of metamorphosis. Increasing TRβ levels, however, coincides with increases in TH levels and reach maximum concentrations during metamorphic climax. Early T_3 response genes, such as basic transcription element binding protein (BTEB) and TRβ may be controlled primarily by TRα (Furlow and Brown 1999). It should be noted, however, that this is a hypothesis and there is no direct evidence to support this theory to date. Genes expressed in the intermediate phase of metamorphosis, such as the basic region leucine zipper transcription factor (TH/bZIP), or late kinetics, including various protease genes, may be controlled by TRβ. [Genes expressed in the "intermediate phase" are defined in opposition to early-acting and late-acting genes during metamorphosis. The term "intermediate phase" refers to mid-metamorphosis, which is similar to the term "prometamorphosis," used by amphibian biologists. The term "late kinetics" refers to genes expressed in late metamorphosis, in accordance with Shi (2000).] Again, no direct evidence has been produced to support this hypothesis. For example, during metamorphosis growing limbs have low TRβ, but display higher expression levels of TRα. The regressing tail displays the opposite TR expression profile (Wang and Brown 1993; Eliceiri and Brown 1994). It has been hypothesized by this this expression pattern that TRα may control cell proliferation in the rapidly growing limbs, whereas TRβ is likely involved in cell differentiation.

399. Using a dominant negative TRα *X. laevis* mutant, Buchholz et al. (2003) demonstrated that the dnTR transgenic line blocked T_3-induced metamorphosis at the onset of prometamorphosis (NF stage 54) and that dnTR inhibited the expression of TH response genes. These investigators used chromatin immunoprecipitation to show that the dnTR bound to the endogenous TH response genes. Binding of the dnTR did not depend on exposure to exogenous T_3. These investigators mutated the ligand binding domain so that the mutant TR could not bind T_3. This resulted in dominant negative activity, since the genes to which the dnTR was bound could not be derepressed. These studies provided the most direct

evidence that T_3-induced metamorphosis requires TRE binding by TR, release of specific co-repressors and subsequent modification of chromatin.

6.7.3 Mechanism of TR Action

400. In general, the TR contains five different binding domains, A/B, C, D, E, F (amino to carboxy terminus), which are reasonably consistent within this class of nuclear receptors (Shi 2000). The amino terminus (A/B domain) of the TRα A/B and TRβ-B2 TR isoforms specifically contains the AF-1 domain, which appears to be involved in T_3-independent recruitment of specific co-activators. The latter isoform is present in mammals, but not amphibians. In mammals, ligand-independent activation of transcription by at least the TRβ-B2 isoform may be mediated by the binding of specific co-factors to the AF-1 region of the A/B domain (Obertse-Berghaus et al. 2000; Yang and Privalsky 2001). DNA binding occurs in domain C. The C domain, which is a generic domain designation for the DNA binding region in the superfamily of nuclear hormone receptors, exists at least in *Xenopus* and in mammals. In *Xenopus,* there are two TRα and two TRβ genes as opposed to mammals, where there is one TRα gene that gives rise to one functional alpha isoform and one TRβ gene that gives rise to three functional beta isoforms of the TR. Alternative splicing of the *Xenopus* TRs gives rise to two different isoforms for each TRβ (designated A1, A2, B1, B2). The C domain is highly conserved amongst nuclear receptors. Domain D is the variable hinge region which contains a nuclear localization signal and influences both DNA binding and transactivation through co-repressor binding (Giguere et al. 1986; Godowski et al. 1988; Hollenberg and Evans 1988; Picard and Yamamoto 1987; Guiochon-Mantel et al. 1989; Zechel et al. 1994; Lee and Mahdavi 1993; Uppaluri and Towle 1995; Puzianowska-Zunicka et al. 1997). Domain E and F are the ligand, or hormone binding and transactivation domains. The carboxy terminus, or region F, contains the AF-2 domain. The AF-2 domain has been found to be a binding site for specific co-activators containing the LXXLL motif of liganded TR (Obertse-Berghaus et al. 2000; Heery et al. 1997; Langlois et al. 1997). TR is presumed to form a heterodimer with the retinoic acid X receptor (RXR). The heterodimer binds to the TH response element in a target gene. In the absence of TH, the heterodimer represses gene transcription, most likely through the recruitment of a co-repressor complex (Horlein et al. 1995; Chen and Evans 1995).

6.7.4 Mechanisms Controlling Pleiotropic Actions of Thyroid Hormones

401. The various mechanisms by which the pleiotropic actions of THs are controlled are evident throughout the various facets of the metamorphic program as described in the preceding sections. These actions are ultimately controlled at six levels, 1) CNS (including hypothalamus), 2) pituitary, 3) thyroid, 4) TH transport, 5) TH elimination (metabolism), and 6) TR. At each of these levels, specific modes of actions could include TH synthesis, TH transport, TH elimination, neuro-endocrine (HPT) axis regulation, and TR expression and/or function. Control at the pituitary level (control of pituitary secretion and control of metamorphosis by these pituitary hormones) is complex since it may involve thyrotropes, corticotropes, and lactotropes (cells that produce TSH, ACTH, and prolactin, respectively). The liver plays a role in T_4 and T_3 homeostasis, notably in TH metabolism and elimination. Similarly, TH transport proteins may play a significant regulatory role in the control of pleiotropic actions of THs.

6.8 Thyroid Hormone Metabolism

402. The metabolism of THs in amphibian larvae have been evaluated using the dual isotope labeling of T_3 and T_4 to minimize the effects of non-specific deiodination and to identify conjugated forms of the THs (Ashley and Frieden 1971). Retention studies indicated that in *R. catesbeiana* larvae, T_4 is retained for a longer period than T_3. Significant differences in metabolism of THs were also found. T_3 was found to be extensively conjugated and excreted as sulfate or glucuronide conjugate. However, T_4 was not extensively conjugated. During metabolism, deiodination was minimal with either TH, except during enterohepatic circulation of T_4. Friesema et al. (1998) also found that THs (T_2>T_3>rT_3>T_4) can be

extensively sulfated via sulfotransferases. Unlike the glucuronide conjugates which are extensively excreted in the bile, sulfate conjugation has been shown to facilitate deiodination of iodothyronines by D1 in mammals. Since D1 is not a primary pathway in amphibians, the role of sulfation in lower vertebrates is presently unknown.

403. Cole and Little (1983) evaluated the role of bile pigments and bilirubin UDPGTs during the metamorphosis of *R. catesbeiana* tadpoles. These investigators found that the major bile pigment in this species was bilirubin Ixα (bilverdin was also measured in the bile) which increased in the bile and the plasma during metamorphosis. UDPGT activity was measured in the livers of premetamorphic larvae; however, naturally metamorphosing tadpoles showed slight increases in activity. T_3-stimulated specimens demonstrated at least 2-fold increases in UDPGT activity.

6.9 Dynamic Relationships among Levels with the HPT Axis

404. Three levels of TH feedback on the HPT axis exist within most anuran species (Shi 2000; Denver 1996; Kaltenbach 1996). Essentially, TH exerts negative feedback on the pituitary and on the CNS, especially the hypothalamus. In cases where TH synthesis is specifically inhibited at the thyroid level, the hypothalamus compensates by releasing CRF, inducing TSH synthesis and release from the pituitary.

6.10 The Role of TH in Amphibian Development and Reproduction

405. The role of TH in amphibian development through metamorphosis is extensively described in the preceding sections and in OECD DRP No. 46, Detailed Review Paper on Amphibian Metamorphosis Assay for the Detection of Thyroid Active Substances (2004). The role THs play in amphibian reproduction is presently unclear, although it is generally thought that the role of TH in adult amphibians is greatly diminished in relation to that during metamorphosis.

6.10.1 The HPT Axis in Relation to the Reproductive Cycle

406. In the adult anuran, the status of the HPT axis varies with season. Adult anurans produce relatively high levels (whole body) of TRH compared to tadpoles (Balls et al. 1985). In fact these levels are great enough that sources outside of the hypothalamus, including the skin, may be involved. As previously discussed in metamorphosing amphibian larvae, the role of TRH is uncertain as CRF is currently proposed to be the primary thyrotropin releasing factor. In *R. pipiens*, TRH levels are lower in the spring and summer than in the autumn or winter (Jackson and Reichlin 1977). Pituitary thyrotrophs and follicular cells of the thyroid demonstrate a similar seasonal pattern (Rosenkilde 1979). GnRH is more potent than TRH in stimulating production of T_4 in *R. ridibunda* in November (Jacobs et al. 1988). However, by February, GnRH is virtually ineffective in inducing increased synthesis of T_4. It is likely that this effect of GnRH is species-specific and temperature dependent. At the level of the thyroid, the seasonal pattern exists; however, interpretation is complicated by marked species differences. In some anurans (*B. bufo*), iodine uptake by the thyroid decreases in the winter, whereas iodine uptake increases during the winter in *R. temporaria* (Ceusters et al. 1978). Thyroid T_3 and T_4 levels in *R. ridibunda* are low during the winter and increase markedly following hibernation (Kuhn et al. 1985). Most importantly, it should be understood that the hypophysiotropic role of systemic TRH is still unknown in amphibians.

407. Following hibernation, the thyroid gland activates and reaches full size immediately prior to reproduction. During reproduction, the thyroid is resistant to TSH stimulation, but reactivates shortly following the reproductive period (Kuhn et al. 1985). *B. bufo* have greater plasma T_4 during hibernation and in the spring breeding period than in the summer and autumn (Rosenkilde 1982). A similar trend was found in *A. tigrinum*. Norris et al. (1977) suggested that TH hormone levels may be inversely related to environmental temperature.

408. In *R. catebeiana*, TSH is present in the pituitary and the thyroid is capable of producing both T_4 and T_3. However, this capacity is substantially diminished compared to metamorphosing larvae (Mackenzie et al. 1978). Since TSH and TH are found in most anuran species, TH would appear to play some specific physiological role in the adult. However, mere presence does not necessarily relate to a specific role. In *X. laevis*, T_4 is not capable of promoting growth in juvenile frogs. However, T_4 is capable of mobilizing energy stores (Nybroe et al. 1985). Increased oxygen consumption in liver cells occurs in *R. tigrinum* as the result of T_4 administration at 25˚C, but not at 15˚C (Packard and Packard 1975). One explanation for the reduced impact of THs in the adult may be a difference in the number of TRs compared to metamorphosing larvae (Galton and Munck 1981). Although liver cell nuclei in adults have similar binding domains for TH as found in larvae and thus similar TH binding characteristics, the actual number of receptors is markedly lower than found in tadpoles. A similar pattern has been found in RBCs of amphibians which spontaneously metamorph. The studies by Galton and Munck (1981) used [^{125}I]T_4 and [^{125}I]T_3. The term "binding domains" refers to the fact that T_4 binds to the receptor in the same capacity; thus the TRs do not bind T_4 differently. These investigators are alluding to a "difference" because of the number of available TRs.

6.10.2 Effects of Gonadal Steroids on Serum TH

409. Unlike corticoids, the role of gonadal steroids on metamorphosis is less certain. Based on an early study by Frieden and Naile (1955) in *Bufo bufo*, estrone enhanced the effect of T_4 on metamorphosis. However, the results of this study have not been demonstrated by other investigators. Rather, the majority of historical studies indicate that estradiol and testosterone antagonize the effects of T_4 in *R. temporaria* (Roth 1941; Roth 1948) and inhibit larval development in *R. pipiens*, *X. laevis*, and *B. boreas* (Richards and Nace 1978; Gray and Janssens 1990; Hayes et al. 1993) *in vivo*. Hayes et al. (1993) found that at 22° C, testosterone and estradiol had no effect on growth or size at metamorphosis, although testosterone induced precocious forelimb emergence. At 27° C, testosterone and estradiol inhibited growth and development, but did not alter the time to forelimb emergence. Gray and Janssens (1990) also found that gonadal steroids did not inhibit the resorption of cultured whole tails *in vitro*. These results suggest that an inhibitory action of gonadal steroids most likely does not occur at the TR level. Gray and Janssens (1990) and Hayes (1997a) suggest that gonadal steroids most likely act at the hypothalamic-pituitary-thyroid axis level. Hayes (1997a) further hypothesized that the most likely mechanism of gonadal steroid inhibition of metamorphosis occurs through the down-regulation of TH levels, and potentially by up-regulating prolactin levels, which as described below also is capable of inhibiting metamorphosis.

410. Several other investigators have evaluated the effects of gonadal steroids on thyroid axis homeostasis and function; and implications on larval growth, development, and metamorphosis (Jacobs et al. 1988; Vandorpe and Kuhn 1989; Hayes et al. 1993). Jacobs et al. (1988) found that plasma concentrations of T_4 were significantly raised following intravenous administration of synthetic luteinizing hormone-releasing hormone (LHRH) in ranids. These investigators concluded that this stimulatory effect was mediated through the hypophysis and suggested a possible correlation between the gonadal axis and thyroid axis. Denver (1988) demonstrated that GnRH can act directly on the frog pituitary to stimulate the release of bioactive TSH. Vandorpe and Kuhn (1989) evaluated the effect of estradiol implants in female *Rana ridibunda* on plasma TH levels and 5'-monodeiodination activity in kidney homogenates *in vitro*. These investigators found that plasma T_3 and TH levels, and the *in vitro* T_3 production in kidney homogenates were significantly decreased, suggesting that estradiol may repress the thyroid axis. Other investigators have evaluated the influence of TH on gonadal steroid activity during metamorphosis (Rabelo et al. 1994; Cohen and Kelley 1996; Robertson and Kelley 1996). Rabelo et al. (1994) found that T_3 enhanced the precocious activation of vitellogenin genes by estradiol in *X. laevis* during advanced metamorphosis between NF stages 58-64. Cohen and Kelley (1996) found that androgen-induced cell proliferation in the developing larynx of *X. laevis* is controlled by TH. These investigators determined that although TH was not required for androgen receptor (AR) mRNA expression in the larynx, cellular

proliferation was enhanced by TH, both *in vitro* and *in vivo*. Further, Robertson and Kelley (1996) concluded that while gonadal differentiation is independent of TH, androgen-sensitive larangeal development, including sexual dimorphism, require exposure to endogenous TH.

6.10.3 Larvae

6.10.3.1 Overview of Early Amphibian Development

411. Early amphibian development has been reviewed by many investigators. In the case of *X. laevis*, Nieuwkoop and Faber (1994) provide excellent descriptions of development through metamorphosis. Similar reviews, including Taylor and Kollros (1946) for ranid species and Rossi (1959) for bufonids are available. In *X. laevis*, hatching occurs ca. 48-50 hours post fertilization, which is relatively quick compared to most temperate ranid species. Organogenesis marks the first 4-d (NF stage 46) of development in *X. laevis*. NF stage 46 also marks the onset of premetamorphosis in *X. laevis*.

412. In the case of amphibian development, premetamorphosis, prometamorphosis, and metamorphic climax are three distinct periods. Premetamorphosis is characterized as a phase of embryogenesis and early tadpole growth in the absence of TH. Morphogenesis includes development of the thyroid gland.

413. During prometamorphosis, amphibians acquire TH synthesis. This phase of development is characterized by concentration of endogenous TH through accelerated TH production. Metamorphic climax is the period in which endogenous TH is at its peak levels and when rapid and drastic morphological changes (i.e., tail resorption) occur.

414. Secondary sexual development in *X. laevis* is appreciably better understood than many of the mechanisms of primary sexual development. As in most vertebrates, secondary sexual differentiation is controlled by gonadal steroids. Responsiveness of a tissue to gonadal steroids can be determined by following the expression of specific receptors. Further, continual secretion of gonadal steroids is required to maintain the secondary sexual characteristics. These patterns are closely followed in secondary structures, including the oviducts in females and the forelimb nuptial pads in males. Oviducts grow in response to estrogen and regress in the absence of estrogen (or in the case of ovariectomy). Clasping behavior and thickening of the nuptial pads are the result of a specific response to androgens. However, both are lost following castration. Some structures or behaviors do not present themselves simply because the appropriate steroids are not present. Alternatively, some characteristics cannot be expressed in adults since the structure was lost during development. For example, the oviduct in developing males regresses presumably as the result of secretion of an “anti-Mullerian hormone”. If castration is performed prior to this developmental process, the oviducts are retained. Generally, the determination of phenotypic sex is capable of proceeding to a point without gonadal influence. Further most species have a default phenotypic sex, female in mammals, male in birds, and female in *X. laevis*. Observations from the former two classes of vertebrate animals led to the assumption that the homogametic sex was the driven default (XX females in mammals and ZZ males in birds) (Adkins 1975). However, the male is the homogametic sex in *X. laevis*. Therefore, the homogametic sex is not necessarily the default phenotype in all vertebrates. As previously discussed, secondary ovarian development involves differentiation of the follicles and oocyte maturation. In *X. laevis*, oocytes are generally divided into six sequential stages ranging from stage I-III which are previtellogenic, stage IV in which vitellogenic growth occurs, and stage V and VI in which final maturation and germinal vesicle breakdown (GVBD) occurs in preparation for eventual ovulation and fertilization (Dumont 1972). Further discussion of GVBD and induction by progesterone and/or androgens will be provided later in this DRP. Although typically dictated by environmental conditions, female *X. laevis* become sexually mature between 12 and 24 months, whereas males generally become sexually mature between 6 and 10 months (Kelley, 1996). In male *X. laevis*, spermatogenesis may occur as early as NF stage 59 (Nieuwkoop and Faber 1994), although this finding has not been confirmed microscopically

(Kelley 1996). Witski (1971) identified spermatocytes two to three months post-metamorphosis. Production of C19 gonadal steroids occurs between stages 59-62 (Kelley and Dennison 1990; Robertson et al. 1991; Kang et al. 1994).

415. The development of gonadal steroid response competence is initiated by TH. May and Knowland (1980) determined that the capacity of larvae to respond to estrogen with induction of the vitellogenin gene begins at NF stage 62 and requires TH secretion. Kawahara et al. (1987) subsequently determined that TH did not directly induce the vitellogenin gene or establish inducibility by estrogen, but rather produced a morphological change in the population of competent hepatocytes in the liver. Further study by Robertson and Kelly (1992) demonstrated that several male secondary sexual characteristics, including development of the larangeal morphology, required TH sensitization for responsiveness to dihydrotestosterone (DHT). Further discussion of the role of TH in conferring gonadal steroid responsiveness during secondary sexual development in *X. laevis* is provided in OECD DRP No. 46, Detailed Review Paper on Amphibian Metamorphosis Assay for the Detection of Thyroid Active Substances (2004) and DRP 4-8 "Amphibian Reproduction and Growth Assay". In short, THs do not appear to act directly on the gonads based on several lines of evidence. First, no TRs exist in the gonad (Kawahara et al. 1991) at this stage of development. Second, TH is not required for continued sensitivity to gonadal steroids or secondary sexual development (Leloup and Buscaglia 1977).

416. Administration of goitrogens, such as thiourea, which block TH production, resulted in skewed sex ratios (100% female) in *X. laevis* (Hayes 1997a; Hayes 1998). In addition, TH has also been shown to directly induce the testosterone receptor in the larynx of developing male *X. laevis* (Cohen and Kelley 1996; Robertson and Kelley 1996). In the sexually dichromatic anuran, *H. argus*, administration of estradiol induces female coloration in both male and female specimens (Hayes 1997a). However, when estradiol is administered concurrently with thiourea, the skewing toward female coloration characteristics does not occur. Hayes (1997a) found that when thiourea, a classical TH synthesis inhibitor, is co-administered with testosterone, induction of gular pouch development does not occur. However, gular pouch development is induced when testosterone is administered alone (Hayes 1997a). However, it should be noted that these experiments could be interpreted as thiourea having a non-specific effect or a requirement for adrogen action. Since a rescue experiment using TH replacement was not reported, conclusive interpretation is not possible.

6.10.3.2 Overview of Morphological Restructuring during Amphibian Metamorphosis.

6.10.3.2.1 Overview of Amphibian Metamorphosis

417. Metamorphosis is a period of substantial morphological change in which an organism alters its mode of living and occurs in all major chordate groups with the exception of amniotes (Dent 1968; Just et al. 1981). Overall, mammals do not have a complex life cycle as do many amphibians. In fact, metamorphosis is developmentally comparable to post-embryonic organogenesis in mammals (Tata 1993). For example, some of the characteristic processes of post-embryonic development in mammals occur in amphibians during metamorphosis (i.e. cell death and proliferation, cell migration and differentiation). Three primary characteristics define metamorphosis, 1) change in non-reproductive structures between a post-hatch or larval state and sexual maturity, 2) form of the larvae enable it to occupy a unique ecological niche different from that used by the adult life stage, and 3) the morphological changes that occur at the conclusion of larval development depend on some environmental stimulus, either external (i.e., temperature or food supply), or internal (hormonal changes). Each of the three classes of amphibians, anurans, urodeles, and caecilians, undergo metamorphosis, although not all species within each class metamorphose. For example, obligatory neotenic urodeles do not metamorphose, and reproduce as an aquatic "adult larvae".

418. Three primary morphological changes occur during metamorphosis, 1) resorption or regression of tissue or organ systems that have primary function only in the larval life stage, 2) the remodeling of larval organ systems to their adult form, which are suitable only for the adult, and 3) *de novo* development of tissues in the adult that are not required by the larvae. These changes are most marked in anuran species, and less obvious in urodeles and caecilians. In each of the three classes of amphibians, metamorphosis is controlled by thyroid hormone (TH), although less is currently known about the role of TH in the metamorphosis of caecilian species. Amphibian metamorphosis has been most widely studied in anurans, primarily due to the dramatic nature of metamorphosis and the ease in use of anuran species in research. However, within the anurans, of which there are nearly 4,000 species (Stebbins and Cohen 1995), metamorphosis has only been reasonably well studied in three species, *Xenopus laevis* (South African clawed frog), *Rana catesbeiana* (bull frog), and *R. pipiens* (Northern Leopard frog).

419. Anuran metamorphosis is separated into three distinct periods, premetamorphosis, prometamorphosis, and metamorphic climax (Etkin 1964; Etkin 1968; and Dodd and Dodd 1976). Premetamorphosis refers to a period of embryonic and early larvae development that takes place without thyroid hormone. Some advanced morphological developments occur during this stage including hind limb bud development. More specific morphogenesis, such as differentiation of the toes and rapid growth (elongation) of the hind limbs, occurs during prometamorphosis. Physiologically, prometamorphosis is characterized by rising concentrations of endogenous TH. The final period is metamorphic climax in which a surge of TH triggers the final processes associated with metamorphosis, including forelimb development and resorption of the tail. Drastic internal transformations at the organ system, tissue, and biochemical levels are also taking place during prometamorphosis and metamorphic climax.

420. From an evolutionary standpoint, amphibians are distinctively separated phylogenetically from other vertebrates. That is, amphibians are separated from other main groups of taxa including mammals, birds/reptiles, and fish. Yet, the vertebrate thyroid axis is generally conserved at both the morphological and molecular levels. Thus, the conserved nature of the thyroid axis enhances the ability to use an amphibian, particularly an anuran, as a general model for evaluating thyroid disruption that can be extrapolated to other vertebrate species.

421. Tata (1998) described amphibian metamorphosis as a unique model for studying thyroid axis function. In most vertebrates, THs have a profound influence on advanced development and growth. Evaluation of the influence of the thyroid axis on fetal development in mammals is complicated by a myriad of maternal factors that modulate the action of TH. On the contrary, amphibian metamorphosis is dependent on the thyroid axis which orchestrates a diverse and well-understood program resulting in physiological and biochemical changes in post-embryonic morphogenesis, selective cell death, and anatomical restructuring in free-living larvae in most anurans. The thyroid axis represents one potential target for environmental chemicals. Environmental agents, toxicants, natural products, and complex mixtures can alter metamorphosis by interacting with the thyroid axis. Further, the complexity of the thyroid axis yields many different possible mechanisms of inhibiting metamorphic processes in amphibians at differing biochemical and molecular levels. Thus, from this end, use of amphibians to screen for thyroid disrupting chemicals or chemical mixtures as a representative chordate is not unreasonable.

422. To date, the debate on endocrine disruptors has mostly revolved around gonadal steroids including estrogens and androgens, because of controversy regarding their possible link to infertility, breast cancer, and lower sperm counts. Thus, the thyroid has received comparatively little attention. Brucker-Davis (1998) reviewed the effects of synthetic chemicals in the environment on thyroid function. This review confirms the hypothesis of thyroid disruption by environmental chemicals in wildlife and supports the need for human population and laboratory animal studies on compounds already identified as thyroid disruptors. In this review, Brucker-Davis (1998) described the effects of over 40 pesticides and 45 industrial chemicals on the thyroid axis.

6.10.3.2.2 Hormones in Metamorphosis

6.10.3.2.2.1 Thyroid Hormone

423. As previously discussed, the primary active THs, T_4 and T_3, are synthesized directly in the thyroid gland (Shi 2000).

6.10.3.2.2.2 Prolactin

424. Similar to the effect of corticoids on metamorphosis (Hayes 1997a), PRL also appears to exert a bimodal effect on development and maturation of amphibians (Shi 2000). However, in the case of PRL, the response is opposite that of corticoids which are capable of inhibiting early development and potentiating TH-induced metamorphosis (Hayes 1997a). In contrast, PRL is currently thought to stimulate development during embryogenesis and premetamorphosis, but inhibit the maturation events associated with metamorphosis. In fact, several investigators (Etkin and Lehrer 1960; Dodd and Dodd 1976; White and Nichol 1981; Kikuyama et al. 1993; Denver 1996) have elaborated on the capacity of PRL to serve as an apparent growth stimulator in amphibians during premetamorphosis, while also inhibiting metamorphosis in anuran species. Also, in contrast to the effect of corticoids on anuran metamorphosis, PRL is capable of exerting its inhibitory influence on metamorphosis *in vitro* (tail explants) (Dodd and Dodd 1976; Tata et al. 1991). These results suggest that the inhibitory effects of PRL on metamorphosis could be mediated at the TR level rather than endocrine regulatory level (Leloup and Buscaglia 1977). In fact, Tata and coworkers demonstrated that PRL is capable of inhibiting induction of the TRβ genes by TH (Baker and Tata 1992; Tata 1997). Wakao et al. (1994) and Han et al. (1997) have also suggested that PRL inhibits the function of the TH-TR complex.

425. Anuran PRL, which was originally difficult to isolate due to the low plasma levels, was first isolated from bullfrogs (Shi 2000). Cloned amphibian PRL was subsequently found to be relatively homologous to mammalian PRL (Yamamoto and Kikuyama 1981; Yasuda et al. 1991; Takahashi et al. 1990; Buckbinder and Brown 1993). Although not considered to be a GH, PRL is a member of the GH gene family and is thus structurally related. PRL in anuran species is produced in the distal lobe of the pituitary gland (Yamamoto et al. 1986; Tanaka et al. 1991). PRL production and secretion is under tight stimulatory and inhibitory control at the hypothalamic level (Kaltenbach 1996; Shi 2000). PRL is transported to various target tissues through the plasma. Low plasma PRL levels have been detected during pre- and prometamorphic stages. However, PRL levels appear to rise to peak levels late in metamorphic climax (Clemons and Nicoll 1977; Yamamoto and Kikuyama 1982; Yamamoto et al. 1986). Interestingly, TRH serves as the primary PRL-releasing hormone in amphibians, whereas, dopamine serves as the primary neurological inhibitor of PRL release. Thus, rather than stimulating the release of TSH (as in mammals), TRH induces the release of PRL and CRF induces the release of TSH.

426. Originally, PRL was thought to serve as an "amphibian juvenile hormone" (Shi 2000) similar to that found in insects. However, work by Baker and Tata (1992) and Tata (1997) suggest that the upregulation of PRL during metamorphosis indicates there is an alternative function during metamorphosis which differs appreciably from the mechanism by which juvenile hormones act (Riddiford 1996). In essence, gene expression profiles for PRL expression at both RNA and protein levels suggest an alternative role for PRL in the control of metamorphic events. Since many of the morphological changes during metamorphosis, such as intestinal remodeling, hind limb digit differentiation, forelimb emergence, and tail resorption (Leloup and Buscaglia 1977; Nieuwkoop and Faber 1994), occur at different developmental stages with differing TH levels, PRL may play a significant role in the coordination of TH-induced amphibian metamorphosis. Further, hypothalamic factors (TRH) do not affect PRL gene expression during the early stages of metamorphosis, but rather rather in its upregulation during metamorphic climax (Shi 2000). *De novo* synthesis of PRL appears to be controlled by TH. Buckbinder and Brown (1993) found

that inhibition of TH synthesis with the classical anti-thyroid drug methimazole repressed PRL gene expression. Conversely, treatment of tadpoles with T_3 leads to precocious upregulation of PRL. Unlike the other hormones and hormonal factors, the anti-metamorphic effect of PRL appears to be exerted at the tissue level rather than in the brain. It is currently thought that PRL inhibits TH activity at the thyroid hormone receptor (TR) level. Although this model needs further confirmation, it appears that PRL interferes with TH binding to TR, thus blocking the action of TH.

427. The current hypothesis regarding the role of PRL during metamorphosis is that PRL appears to control the high concentrations of TH present during metamorphic climax so that sequential transformation of different tissues can be systematically coordinated (Shi 2000). This potential role is significant in tadpole-frog transformation, since different tissues/organ systems require differing TH levels at different times during metamorphosis. For PRL to exert this effect, it must act directly at the tissue level and relatively early in the TH signal transduction process (Leloup and Buscaglia 1977). Tata and coworkers demonstrated that PRL is capable of inhibiting induction of the TRβ genes by TH (Baker and Tata 1992; Tata 1997). Wakao et al. (1994) and Han et al. (1997) have also suggested that PRL inhibits the function of the TH-TR complex.

428. Another hypothesis is that PRL interacts with a membrane bound receptor that initiates a cascade of biochemical events that give rise to transcription factors known as Stats (signal transducers and activators of transcription). Interaction of Stats and TR leads to the inhibition of TR and thus, blocks TH-induced metamorphosis (Kanamori and Brown 1992). Based on this model, the effects of PRL on TH action are tissue-dependent, because receptor and Stat levels likely differ in the different cell types. This may provide a method of coordinating systematic transformation of different tissues during metamorphosis. Overall, this discussion demonstrates the importance of other related hormonal systems in the control of metamorphosis.

429. More recently, Favre-Young et al. (2000) demonstrated that TRβ/RXR transfected cell lines (HEK293) significantly repressed PRL-dependent Stat5a- or Stat5b-induced reporter gene expression. Further, over-expression of the TRβ/RXR complex resulted in increased nuclear localization of Stat5a. These investigators concluded that TRβ/RXR modified the subcellular distribution of transcriptional activator Stat5a, thus providing regulation over this PRL-mediated signaling pathway.

430. Melatonin, produced by the anuran pineal gland and somatostatin appear to be capable of altering anuran metamorphosis (Shi 2000). Both factors inhibit TSH secretion and are capable of retarding metamorphosis (Denver 1996). Melatonin may also inhibit metamorphosis through the induction of PRL pathways (Rose and Rose 1998). On the contrary, gonadotropin-releasing hormone (GnRH) has been found to increase TH levels in axolotls and frogs, and thus has the potential of accelerating metamorphosis (Shi 2000). Limited information is available on these and other hormones and the understanding of their roles in metamorphosis remains unclear.

6.10.3.2.2.3 Corticosterone

431. In general, the relative importance and capacity of corticosteroids in enhancing TH-induced metamorphosis in amphibians has been purported by several sets of investigators (Kaltenbach 1985; Kikuyama et al. 1993; and Hayes 1997a). In amphibians, the interrenal gland is responsible for the production of corticosteroids and receives direct input from the pituitary via adrenocorticotropin (ACTH). In turn, two primary corticoids are produced and secreted by the anuran interrenal gland: 1) corticosterone, and 2) aldosterone (Cartensen et al. 1961; Macchi and Phillips 1966; and Kikuyama et al. 1993; Shi 2000). Interestingly, several investigators have demonstrated that the major corticoid levels in plasma in metamorphosing anurans follow the pattern of rising plasma TH levels in metamorphosing tadpoles (Jaffe 1981; Krug et al. 1983; Jolivet-Jaudet and Leloup-Hatey 1984; Kikuyama et al. 1986; Kikuyama et al.

1993; Hayes 1997a). Experimental evidence supporting the role of corticoid hormones in the induction of metamorphosis ranges from basic fundamental studies to complex experiments. Further, the time courses of aldosterone and corticosterone were reported for the complete development of *X. laevis* by Kloas et al. (1997).

432. For example, Kaltenbach (1985) and Kikuyama et al. (1983) found that exogenous administration of corticoids via the culture media enhanced tail resorption of premetamorphic tadpoles. Similar responses in cultured anuran tails also have been noted as the result of exogenous corticoids (Kikuyama et al. 1983; Hayes et al. 1993; Hayes and Wu 1995a; Hayes and Wu 1995b; Hayes 1997a). Several different tissues in the metamorphosing anuran appear to be responsive to the impact of corticoids on TH action including: 1) the limbs (Galton 1990; Kikuyama et al. 1993; Hayes 1997a), and 2) the skin (Shimizu-Nishikawa and Miller (1992). Further, corticoid receptor sites have been identified in the metamorphosing anuran tail and determined to be important in the control of metamorphosis (Woody and Jaffe 1984 and Yamamoto and Kikuyama 1993).

433. As an alternative to exogenous corticoid supplementation, the influence of inhibiting the synthesis of endogenous corticoids on metamorphic processes was also evaluated (Kikuyama et al. 1982). In essence, results from these studies indicate that inhibition of corticoid synthesis using Amphenone B is capable of reducing the efficacy of exogenous TH supplementation to thiourea-induced thyroid repressed amphibians. This study suggests that TH and corticoids work in concert to influence amphibian metamorphosis.

434. A study by Hayes (1997a) suggests that corticoids may operate under a dual mode of action based on the stage of anuran metamorphosis. Based on these studies (Hayes 1997a), corticoids appear to slow development during early embryogenesis. Prior to and during the early stages of prometamorphosis, endogenous TH levels are low. As TH levels begin to rise with the onset of metamorphosis, corticoids enhance the capacity of TH to induce metamorphosis, although a clear mechanism is not yet known. Much of the evidence supporting the role of corticoids in amphibian metamorphosis is based on *in vitro* studies involving cell and organ cultures. Based on these studies collectively, corticoids appear to exert negative feedback at the pituitary and hypothalic levels in anurans (Denver and Licht 1989; Galton 1990; Nishikawa et al. 1992; Shimizu-Nishikawa and Miller 1992; Gancedo, et al. 1992; Denver 1993; Schneider and Galton 1995; Tata 1997; Hayes 1997a).

435. The influence of corticoids on TH-induced metamorphic events has also been observed at the cell and molecular level (Galton 1990; Kikuyama et al. 1993; Hayes 1997a). For example, maturation of the skin which occurs during the metamorphic transition of the larvae to an adult involves the expression of adult keratin genes in the epidermis of *X. laevis*. Under normal physiological conditions, up-regulation is controlled by TH. However, corticoids have also been shown to potentiate the response of these genes to TH. Current research suggests that corticoids act through a nuclear receptor, the glucocorticoid receptor (GR). The GR appears to be similar to classical nuclear-based steroid receptors which essentially belong to the same superfamily of receptors that includes TH receptors (Evans 1988; Green and Chambon 1988; Mangelsdorf et al. 1995). Thus, as with most steroid hormones, corticoid effects are induced at the transcriptional level. The *X. laevis* GR has been cloned (Gao et al. 1994) and developmental expression and hormonal regulation of both the GR and TR in *X. laevis* has been profiled (Krain and Denver 2004).

436. In summary, the synthesis and secretion of endogenous corticoids are under the direct or indirect control of TH, ACTH, and CRF. Based on the work of Denver and Licht (1989), CRF appears to have dual functions, stimulating the release of both TSH (thyrotropes) and ACTH (corticotropes) from two different regions of the pituitary. Conversely, the role of TRH in metamorphosis which is the primary thyrotropin releasing factor in most mammals, is currently thought to be insignificant (Shi 2000). Overall, physiological synthesis and secretion of corticoids play an important role in anuran metamorphosis.

6.10.3.2.3 Role of TH in Larval Organ Resorption

437. Morphological changes that occur during amphibian metamorphosis have been extensively described and various reviews exist regarding these drastic changes in anatomy (Dodd and Dodd 1976; Hourdry and Dauca 1977; Gilbert and Frieden 1981; Fox 1983; Balls and Bownes 1985; Yoshizato 1989). Essentially, three primary changes in tadpoles take place during metamorphosis in order to transform almost all of the tadpole organs to their adult form (Shi 2000). The first change involves complete destruction or digestion of tadpole-specific organs. The most obvious example of such a resorption process is the loss of the tail during metamorphic climax. The second change involves *de novo* development of new tissues from newly produced and proliferated cells. As with many embryological processes these newly produced cells differentiate, ultimately leading to tissue morphogenesis and development of the digits of the hind limbs. Finally, restructuring of existing organ systems into their adult forms occurs creating the liver, lungs, and intestine. These processes allow the metamorph to either adapt to a new terrestrial environment or to enter adulthood in species that remain aquatic. For the sake of brevity, only morphological features that are relevant to the development of amphibian metamorphosis assays will be discussed in this DRP.

438. Of the organ systems resorbed during metamorphosis, two systems degenerate completely: the tail and the gills. Of these two organ systems, resorption of the tail has been the most widely studied (Dodd and Dodd 1976; Houdry and Dauca 1977; Gilbert and Frieden 1981; Fox 1983; Balls and Bownes 1985; Yoshizato 1989). All tissues that comprise the tadpole tail are resorbed during metamorphosis, including an epidermis, connective tissue, muscular tissue, blood vessels, and the notochord (Shi 2000). Typically, tail resorption occurs at the onset of metamorphic climax around NF stage 62 with the loss of cross-striations of the myofibrils and disintegration of sub-cellular structures, such as the mitochondria (Weber 1964; Dodd and Dodd 1976) and concludes around stage 65 to 66 with the complete disintegration of the tail fin.

439. Condensation and histolysis primarily contribute to tail resorption (Yoshizato 1989). The loss of tail length during metamorphic climax results from condensation. Water loss, in turn, causes alteration of the cellular organization of the tail tissue resulting in compaction of the cells and extracellular matrix (Frieden 1961; Lapiere and Gross 1963; and Yoshizato 1989; Yoshizato 1996). Extensive studies have focused on histolysis as a potential mechanism of condensation, but the specific mechanism has not yet been clearly identified (Kerr et al. 1974; Kinoshita et al. 1985; Yoshizato 1989). The cells of the tail undergo programmed cell death or apoptosis, and the extracellular matrix is degraded by various enzymes. This process is consistent with apoptosis in other vertebrates. The tail is genetically pre-determined to resorb, requiring only sufficient levels of TH to initiate the process.

6.10.3.2.4 Role of TH in Adult Organ Development

6.10.3.2.4.1 Limbs

440. The classic example of *de novo* development is the limbs. Hind limb buds are first visible in *X. laevis* at stage 46 and continue to grossly develop until stage 54 without the assistance of TH. However, between stages 54 and 58, plasma TH levels rise and induce the differentiation of the limb bud cells allowing morphogenesis to form the specific features of the hind limb and toes. The forelimb develops in a similar pattern with the undifferentiated limb bud developing without endogenous TH.

6.10.3.2.4.2 Gut, Nervous System, Intestinal System, and Lungs (Gills)

441. The majority of organ systems are present in both the tadpole and the adult anuran; however, most require some form of modification in the adult (Shi 2000). The liver undergoes little overt

morphological change; however, extensive biochemical changes take place during metamorphosis. Fine structural changes take place in the cells of the liver during the early and intermediate stages of metamophosis, including increases in the size of mitochondria, endoplasmic reticulum, and Golgi complexes. Changes also take place during the later stages of metamophosis that increase the biosynthetic capacity of the liver during metamorphosis, including increases in the number of nucleoli and the heterochromatic nature of the nuclei. On a biochemical level, hepatocytes within the liver convert from ammonotelic to ureotelic metabolism (Atkinson 1994; Atkinson et al. 1996; Chen et al. 1994). The nervous system is also restructured to accommodate adult physiology (Kollros 1981; Fox 1983; Gona et al. 1988; Tata 1993, Shi 2000). At the gross morphological level, changes in the shape of the interrenal gland, diencephalon, medulla, and restructuring of neurons in the cerebellum occur during metamorphosis (Gona et al. 1988).

442. At the neuronal level, one of the most dramatic changes includes the genetically programmed regression of the various specialized cells. These cells include a specialized pair of giant neurons, Mauthner cells and Rohon-Beard neurons, which regress or disappear completely, respectively, during metamorphosis (Hughes 1957; Moulton et al. 1968). In a recent study by Cohen et al. (2001), these investigators found that the antiapoptotic protein Xr11 prolonged survival of Rohon-Beard neurons and reduced morphological change to Mauthner cells. However, Xr11 was not effective in controlling the alterations and ultimate disappearance of other neurons, including spinal cord motor neurons. On the contrary, Purkinje cells, lateral motor column neurons, and the dorsal root ganglia neurons further differentiate during metamorphosis (Hoskins 1990).

443. The tadpole intestine is proportionately longer, but more simplistic than the adult anuran intestine, which resembles most vertebrate intestines in terms of structure and function (Shi 2000). The tadpole intestine is comprised of a single epithelial layer surrounded by thin layers of muscle and connective tissue (McAvoy and Dixon 1977; Kordylewski 1983; Ishizuya-Oka and Shimozawa 1987; Shi and Ishizuya-Oka 1996). Remodeling of the intestine during metamorphosis creates a shorter, but substantially more sophisticated epithelium with numerous microvilli, brush boarders, and ultimately, substantially increased luminal surface area. Thus, the increased microstructure of the intestine developed during metamorphic climax increases the effectiveness and efficiency of absorption in the intestine, thereby reducing the need for the proportional length found in the tadpole intestine.

444. In most anurans, the gills are internal and serve as the primary respiratory organs prior to the development of the lungs. However, because anuran larvae remain aquatic, complete resportion of the gills does not occur until late, typically during metamorphic climax during which time the tail is resorbing (Atkinson 1981). In *X. laevis*, resoprtion of the gill tissue begins around stages 61/62 (Atkinson and Just 1975; Atkinson 1981). During this time, extensive morphological changes occur, and the gill epithelium separates from the vascular network while this network is undergoing histolysis. By stage 64 melanocytes begin to take the place of the gill tissue resulting in a black appearance. Rapid degeneration of the gill tissue occurs until the gill tissue resorbs completely.

6.10.3.3 Biochemical Changes

445. The cellular and biochemical changes that occur in anurans during metamorphosis can be divided into at least six general areas: 1) molecular and biochemical activities associated with programmed cell death or apoptosis, 2) shift from ammonotelism (ammonia excretion) to ureotelism (urea excretion), 3) increase in serum protein levels, 4) changes in hemoglobin isoforms, 5) alterations in digestive enzymes, and 6) changes in the respiratory system (Shi 2000). The role of apoptosis in the selective resorption of tadpole tissues was discussed in the previous section. Many of the genes involved in programmed cell death have been isolated and characterized largely due to genetic studies in *Caenorhabditis elegans*. At least three execution genes, seven engulfment genes, and one degradation gene are involved in apoptosis in

the nematode *C. elegans*, which represent the three primary processes in selective cell death (Yuan et al. 1993; Alnemri et al. 1996; Cryns and Yuan 1998). Genes participating in execution of apoptosis and subsequent steps are most likely common in biological organisms where cell death takes place (Ellis and Horovitz 1986). Homologs to the *C. elegans* and mammalian genes are currently being studied in amphibians. On the other hand, signal transduction genes participating in the early steps leading to apoptosis, such as induction by TH, may vary in different species. A primary feature of apoptosis involves fragmentation of chromatin which is exploited as a means of evaluating apoptosis by TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling). TUNEL is capable of marking apoptosis prior to the fragmentation of the nucleus and the cytoplasm (Gavrieli et al. 1992). The initiation of apoptosis is dependent on THs, and increasing concentrations of T_3 (5 to10 nM) induce an increasing response, demonstrating dose-dependence. Classical inhibitors of apoptosis, including ATA (aurintricarboxylic acid), the protein sequence Z-VAD (Z-val-ala-asp-fluoromethalketone), and the antibiotic CsA (cyclosporin A), are capable of inhibiting epithelial apoptosis (Su et al. 1997; Shi et al. 1989). Apoptotic bodies in the tail can be observed as early as NF stage 59 in *X. laevis* (Shi 2000).

446. Anuran tadpoles primarily excrete nitrogen waste in the form of ammonia (ammonetelism) during premetamorphosis (Munro 1953). During the onset of metamorphic climax, ammonia excretion decreases and urea (ureotelism) excretion increases. In most juvenile anurans, at least 75% of nitrogen waste is comprised of urea (Brown et al. 1959). *X. laevis* represents a primary exception to the excretory conversion to ureotelism. Since *X. laevis* maintains an aquatic life history as an adult, it primarily excretes ammonia under normal conditions (Munro 1953). Transient increases in urea during prometamorphosis are typically detected (Underhay and Baldwin 1955). However, restrictions in water supply induce a drastic increase in urea excretion, suggesting that *X. laevis* has similar excretory conversion capabilities as the terrestrial anurans (Balinsky et al 1961).

447. During the process of tail resorption, upregulation of a series of proteases, acid and alkaline hydrolases, and ribonucleases occurs (Shi 2000). Upregulation of the degradative enzymes is regionally specific in the tail. For example, acid phosphatase levels in the dorsal fin region, which resorbs first in this process, are dramatically elevated in this region during this time. Similar lysosomal hydrolases are upregulated in the intestine during re-modeling. In each case, TH has been shown to control up and down-regulation of these degradative enzymes during metamorphosis. An effective description of the degradation enzyme genes regulated by thyroid hormone in anurans appears in Shi (2000).

448. In most anuran species, serum protein levels dramatically increase during metamorphosis (Shi 2000). The ratio of serum albumin to globins is markedly elevated during TH-induced metamorphosis. Other plasma proteins that increase during metamorphosis include ceruloplasmin, transferrin, and carbonic anhydrase (Inaba and Frieden 1967; Frieden and Just 1970; Wise 1970). The increase in serum proteins is thought to play an adaptive role as the tadpole transforms into the frog. For example, albumin provides and maintains osmotic requirements, as well as circulatory transport requirements warranted for a terrestrial habitat (Frieden 1968; Weber 1967; Broyles 1981).

449. Anurans undergo changes in hemoglobin synthesis during development similar to mammals and birds (Shi 2000). However, in anurans, only one primary change occurs compared to other animals, which typically undergo several changes in synthesis patterns, including complete replacement of globulin chains (Weber 1996). Hemoglobin in tadpoles is independent of pH and has a markedly greater affinity for oxygen than frog hemoglobin, which has lower oxygen binding affinity and is subject to the Bohr Effect (decreased affinity with decreasing pH) (McCutcheon 1936; Riggs 1951; Frieden 1961). The greater affinity of tadpole hemoglobin for oxygen most likely allows for adaptation to low oxygen environments characteristic of the aquatic tadpole habitat. Changes in hemoglobin structure occur around metamorphic climax, although larval hemoglobin persists for some time in metamorphosed frogs to allow for adequate adaptation to the change in the environment (Just and Atkinson 1972; Weber et al. 1991). Adult frogs

require hemoglobin with lower oxygen affinity to facilitate terrestrial life, which has more rapid and extensive oxygen requirements (Bennett and Frieden 1962; Dodd and Dodd 1976).

450. In addition to an increase in plasma proteins, the metabolic capacity of the liver markedly changes in the metamorphosing anuran. During metamorphosis, drastic increases in nucleic acid and protein synthesis result in large increases in several enzymes including, catalase, uricase, phosphatases, and the urea cycle enzymes (Shi 2000). Of these enzymes, the urea cycle has been the most widely studied. Upregulation of these enzymes has been observed during anuran metamorphosis and induced by TH as the direct result of *de novo* protein synthesis (Brown and Cohen 1958; Brown et al. 1959; Paik and Cohen 1960; Cohen 1970; Dodd and Dodd 1976). Although each of the four primary enzymes associated with the urea cycle increase at least several fold, the mitochondrial enzyme carbamyl phosphate synthetase actually increases nearly 30-fold during metamorphic climax. Cytosolic arginase catalyzes the conversion of arginine to urea waste, and ornithine (Figure 3-2) increases nearly 5-fold in activity in the livers of metamorphing anurans.

6.10.3.4 Overview of Amphibian Larval Immune Function

451. Consistent with other vertebrates, the major histocompatibility complex (MHC) which represents a cluster of genes encoding products central to major functions of the immune system is also present in amphibians. Expression of MHC class I and II antigens early in development is critical for the development of T cells capable of discriminating self from non-self. In *X. laevis*, class I antigens are virtually absent from larval tissues until metamorphic climax (Rollins-Smith et al. 1997a). Overall, the characteristics of the immune system in larval *Xenopus* are markedly different than that found in the adult with metamorphosis triggering the change in systems (Rollins-Smith et al. 1997b). Changes during this transition actually create increased susceptibility during metamorphosis due to elimination of larval lymphocytes which decrease the possibility of attack on the newly developed structures (adult-specific antigens), but create an immunocompromised animal for a short period of time until metamorphosis is complete (Rollins-Smith 1998).

452. Rollins-Smith et al. (1997a) evaluated the production of class I antigens during metamorphosis finding that a slight induction of splenocytes and erythrocytes occurred during prometamorphosis. The amount of class I antigens increased dramatically during metamorphic climax. Neither acceleration nor inhibition of metamorphosis altered the timing of class I antigen expression. Further, expression was not increased when TH was administered to metamorphosis-inhibited specimens suggesting that the expression of class I antigens was not directly cued by TH. However, unbound glucocorticoids were associated with a natural decline in total lymphocytes, lymphocyte viability, and mitogen-induced proliferation (Rollins-Smith et al. 1997b). A reduction in total lymphocyte numbers appears to be the direct result of corticoid-induced apoptosis. Thus, corticoids remove unnecessary lymphocytes to allow for the development of immunological tolerance to the new adult-specific antigens that appear as the result of metamorphic change. However, exposure to an environmental stressor, such as drying of a temporary pond, can induce metamorphosis at a smaller than normal body size, which can lead to an abnormally small adult animal with immune system deficiencies. Thus, the organism could potentially be at greater risk of infection (Rollins-Smith 1998). Although the role of THs in inducing change in the immune system during metamorphosis appears to be minor in terms of class I antigens, Ruben et al. (1989) found that T_3 stimulated an increase in the number of cells in *X. laevis* capable of binding to an interleukin 2 receptor antibody. Therefore, some metamorphic-based changes in immune function may be controlled by THs.

6.10.3.5 Overview of Neuroendocrine Stress System

453. The primary neurohormonal stress pathway in amphibians involves CRF (Denver 1997). Environmental stress, such as habitat desiccation, results in rapid metamorphosis induced by a CRF-

mediated pathway. This process can be simulated in the laboratory by inducing habitat drying which stimulates the production and release of CRF, or by injection of CRF (Denver 1997).

454. As discussed previously, CRF is thought to be the primary thyrotropin releasing factor in amphibians. CRF is responsible for increasing levels of TSH and THs and inducing metamorphosis, which in some cases is precocious (Denver 1997). Interestingly, CRF of fetal or placental origin has been shown to induce parturition, including inducing pre-term delivery in cases of fetal or maternal stress in mammals. Thus, Denver (1997) suggested that this process may represent an evolutionarily-conserved means of responding adaptively to a deteriorating larval/fetal habitat.

6.11 Evaluating Thyroid Disruption in Anurans

6.11.1 Potential Sites of Action

455. EDCs could potentially affect the thyroid axis at six levels, 1) CNS (including hypothalamus), 2) pituitary, 3) thyroid, 4) TH transport, 5) TH elimination (metabolism), and 6) TR. Specific modes of actions of thyroid axis disruptors could potentially include alteration of TH synthesis, TH transport, TH elimination, neuro-endocrine axis regulation, and TR expression and/or function. A summary of potential modes/sites of EDC action on the thyroid axis, in relation to endpoints possibly useful in measuring thyroid disruption, is provided in the Detailed Review Paper on Amphibian Metamorphosis Assay for the Detection of Thyroid Active Substances, OECD Series on Testing and Assessment, No. 46 (OECD 2004). The effect at the pituitary level is complex since it may involve thyrotropes (TSH), corticotropes (ACTH), and lactotropes (PRL). In addition to the thyroid gland, the interrenal gland may also be a site of EDC action, which could potentially impact metamorphosis. Thus, the impact of potential EDCs on metamorphosis may occur at multiple levels.

456. In addition, other physical environmental factors such as temperature and water level (densities, resource declines, increased temperature, increased predator density, and changes in pH and dissolved oxygen) may also alter metamorphosis. Hormonal factors outside the thyroid axis, such as the corticotropes, may also affect metamorphosis. The liver should not be overlooked since it plays a role in T_4 and T_3 homeostasis, notably in TH metabolism. Similarly, TH transport proteins, including CTHBPs, should also be considered. The complexity of metamorphosis and control by the neuroendocrine system must be strongly considered in the design of appropriate test methods. Since the objective of the test method is to screen for thyroid axis disruption, a rapid, high throughput biochemical test or molecular test would likely be more advantageous. However, biochemical measurements and molecular tests alone might not provide adequate information on the morphological effect of the EDC on metamorphosis at the whole organism level. Incorporation of a high throughput biochemical or molecular assay within a short-term morphological method would be advantageous. It is crucial that the methodology used, demonstrate diagnostic power by distinguishing between non-thyroid and thyroid-related delays in developmental progress.

6.11.2 Relevant Endpoints in Anurans

457. Apical morphological endpoints are useful in that they demonstrate an organism-level effect that integrates all aspects of the toxicological process. While there is value in apical morphological endpoints, they are limited because: 1) they are not necessarily diagnostic of a particular mode of action, 2) they are generally less sensitive than sub-organismal endpoints, 3) they are the slowest of the endpoints to manifest effects, and 4) they may be difficult to use in terms of inter-species extrapolation. For example, Tietge et al. (2005) have demonstrated that histological analysis of the thyroid gland of organisms that have been exposed to several TH inhibitors can detect substantial thyroidal hypertrophy and hyperplasia in 8 days at concentrations where there is no effect on the apical morphological endpoints. These observations suggest

that, based on apical endpoints, one would conclude that the thyroid pathway is unaffected, when in fact there is clear evidence of a thyroid-specific effect at a histological level. The interpretation of this pattern of responses is that the thyroid axis is inhibited, but the mechanisms that underlie homeostasis are able to compensate at a dose which results in incomplete inhibition. More specifically, partial inhibition of TH synthesis results in depressed TH levels, which is sensed by the CNS and results in TSH release which, in turn, stimulates thyroidal hyperplasia and hypertrophy. This response compensates for low TH levels by up-regulated synthetic pathways.

458. Another problem with apical morphological endpoints is that they tend to be divergent and do not lend themselves to inter-species extrapolation. Endpoints which address more central and conserved processes are more desirable because they do lend themselves to inter-species extrapolation. For example, several chemicals are known to inhibit TH synthesis via different mechanisms. The same mechanisms are apparently affected similarly in both mammals and amphibians (e.g., inhibition of iodide uptake by perchlorate, inhibition of thyroid peroxidase by methimazole). The manifestation of these inhibitory mechanisms in an anuran at the apical level would be inhibition of metamorphosis based on a morphological response, such as tail resorption. It would be difficult to use this response to predict effects in mammals, where tail resorption does not occur. Therefore, if the subject protocol is to be used as a generalized vertebrate model, then apical endpoints are insufficient and endpoints with more diagnostic power are clearly necessary.

459. Three additional classes of endpoints that should be considered for this assay: 1) molecular endpoints, 2) biochemical endpoints, and 3) histological endpoints. Classical toxicology tests focus primarily upon tissue and organism-level effects, which are often insufficient for discriminating the modes/mechanisms of action (MOA). A fundamental understanding of MOA, however, is critical to the ability to extrapolate toxicological effects among species and chemicals, and across biological levels of organization. This necessitates the development of tools capable of linking tissue and organism-level effects to MOA. Monitoring gene expression is one approach commonly used to link whole organism changes to specific MOA. However, traditional methods in molecular biology have generally utilized a "one gene in one experiment" approach and, as a consequence, the through-put is very limited and resource-intensive when more than a few genes are monitored. In the past several years, a new technology, called DNA array analysis, has been developed to monitor a large number of genes simultaneously (Nuwaysir et al. 1999; Khan et al. 1999; Debouck and Goodfellow 1999). Application of this tool to the study of thyroid toxicology could allow investigators to simultaneously monitor the expression of many thyroid related genes at multiple points within an organism. This approach could potentially provide insight as to where in the organism or tissue toxicity is occurring, and which genes might serve as indicators of exposure and effects. However, the transcription of a gene does not necessarily reflect the chemicals MOA unless additional information is collected that provides a link to the adverse effect induced. More specifically, this link cannot be made unless specific known genes for well-characterized pathways are monitored in the arrays. Further work will be required if the combination of DNA arrays and whole organism testing are able enhance our ability to link functional changes in the organism to specific MOAs.

460. Biochemical endpoints, like molecular endpoints, provide useful information on MOA and are, thus, important in developing a protocol that provides data that are diagnostic of a thyroid-specific effect and that can be extrapolated to other vertebrates. The most commonly used method to assess T_3 and T_4 status is RIA. RIA determinations on organisms exposed to a chemical represent the minimal data necessary to evaluate inhibition of T_4 synthesis and metabolic conversion of T_4 to T_3 via deiodinase activity. As previously indicated this merthod requires adequate tissue extraction. Other methods should be considered that permit the analysis of synthetic precursors of T_4, as well as metabolic products that are the result of elimination pathways (e.g., deiodination, glucuronidation, sulfation). Several HPLC-based methods have been utilized to achieve separation of such compounds, but they often rely on the use of

radiolabeled iodine for detection. Alternative detection methods should be further investigated and possibly included as routine analytical methods to augment the utility of a Tier I protocol. Finally, histological analysis of the thyroid must be included as an endpoint at this time because it confers diagnostic specificity to the assay that is unattainable using apical morphological endpoints alone.

461. Histological endpoints represent a direct evaluation of effect of thyroid gland impairment at the tissue level. Based on studies conducted to date (Degitz et al. 2005), histological evaluation of the thyroid gland provides a relatively straightforward means of evaluating a direct impact on target tissues such as the thyroid gland.

462. In summary, endpoints which confer diagnostic specificity are needed in order to accurately determine whether or not the thyroid pathway is the target of a test chemical. This is needed, not only to properly characterize the chemical for potential regulatory action, but to provide a basis for interspecies and interchemical extrapolation. Although the molecular and biochemical endpoints suggested herein require additional research and development, their successful implementation will reduce the time and cost associated with conducting an assay, and will improve the quality and utility of the data. Ultimately, this will help reduce testing needs in the future.

6.11.2.1 Whole Animal Assays

6.11.2.1.1 Morphological Measures

463. Morphological measures of metamorphosis and thyroid status include tail resorption, limb emergence and development, skin development, and skin coloration (*Hyperolius*). However, it should be noted that *Hyperolius* is not a common species, and use of this species in a screening assay will most likely be impractical. Protocols ultimately developed to morphologically mark thyroid impairment may include any applicable endpoint discussed in the following sections, and should not be limited to one endpoint if possible.

6.11.2.1.1.1 Tail Resorption

464. Tail resorption occurs in anuran species during metamorphic climax and is triggered by a surge of TH. Late prometamorphosis (NF stage 58-61) and metamorphic climax (NF stage 61-66 with completion of metamorphosis at NF stage 66) in *X. laevis* occurs roughly over a 16-18 day period and incorporates stages 58 to 66. Tail resorption can be monitored in culture using digital photography and measuring the tail lengths with a scanning digitizer. A rate of tail resorption can be determined from the mean length data obtained. Abnormal tail resorption can also be monitored during this process. Specimens can be selectively preserved throughout the process of tail resorption and evaluated morphologically. In terms of culture, different approaches may be considered. For example, the specific process of tail resorption can be monitored by culturing tadpoles at relatively low densities such as in multiple replicates for 16 to 18 d from stage 58 to 66 during which time the rate of tail resorption is measured (Fort et al. 2000).

465. Based on this information, several factors relating to the measurement of tail resorption and the interpretation of the results require consideration. First, the rate of tail resorption is naturally variable in whole organism culture (Fort et al. 2000; Fort et al. 2001b), which reduces the sensitivity and predictability of this endpoint. Second, this process occurs in the later stage of metamorphosis, when the thyroid is fully active and thyroid hormones are at their peak early in the climatic period. However, when used with other morphological and biochemical or molecular endpoints, such as TH measurement and TR gene expression, this endpoint could be considered. Issues regarding exposure design, including the use of flow-through systems, need to be addressed.

6.11.2.1.1.2 Limb Development

466. As previously discussed, early hind limb bud development (emergence) occurs prior to thyroid activity in the developing anuran tadpole. However, hind limb differentiation and forelimb development occur during the prometamorphic phase of metamorphosis. Thyroid dysfunction impairs the process of limb differentiation, but not limb bud emergence. Selecting an exposure window that encompasses hind limb differentiation would require exposure from stages 54 to 60. A longer-term test format described by German investigators, incorporates limb differentiation as an endpoint. The only standardized test method that evaluates limb development was evaluated by Fort and Stover (1996) and Fort et al. (1997) using *X. laevis*. However, this modified FETAX assay (ASTM 1998) evaluated only hind limb development, initiating exposure at an early blastula stage and completing exposure around 30 days at stage 54. Thus, this design did not address the effects of thyroid dysfunction on limb differentiation, and toxicant exposure was longer than necessary since it incorporates a substantial period of premetamorphosis. However, a modification of the assay might be considered that expresses *X. laevis* from stage 51 (limb bud stage) to stage 54, at which time the hind limb is reasonably well differentiated.

467. The morphology, rate, and extent of limb differentiation can be monitored in a similar manner as described for tail resorption. However, in this case the occurrence of abnormal limb development, including asymmetrical differentiation, should be monitored, thus requiring additional specimens for histological examination, CAT scan, and x-ray analysis.

6.11.2.1.1.3 Skin Development

468. During metamorphosis, substantial changes to the skin in terms of protein structure, keritinization, and pigmentation occur (Watanabe et al. 2003; Kaltenbach et al. 2004). Changes in skin structure have already been discussed; however, changes in pigmentation also occur that change a transparent tadpole such as *X. laevis* to a frog with pigmented, non-transparent skin. Classical thyroid inhibitors, such as thiourea, also inhibit pigmentation by blocking melanin synthesis. Melanin distribution in skin structure can be evaluated in *Xenopus* under normal light microscopy. Unlike larval skin, metamorph skin possesses well-formed melanocytes containing a relatively dense distribution of melanin. Staining is only required to evaluate the neurological status of the pigmentation process. For evaluation of epidermal structure and keritinization, a standard eosin or hemotoxylin/eosin can be effectively used.

469. Immunohistochemical techniques can be used to distinguish the presence of specific proteins that mark the newly developing frog skin. Therefore, skin maturation is a potential valuable endpoint. Like the endpoints previously discussed, this endpoint is best served with a battery of other metamorphosis-based morphological endpoints in a longer-term exposure design (Watanabe et al. 2003; Kaltenbach et al. 2004).

6.11.2.2 Pathology Endpoints

470. In most cases, thyroid dysfunction, such as goiter or myxedema, manifests changes in the morphometry of the thyroid. For example, tadpoles exposed to the goitrogen methimizole develop enlarged thyroid glands that are visible under low magnification in the transparent *Xenopus* tadpole (Fort et al. 2001a, b). In this case, the thyroid gland can be digitally photographed and the size quantified by photodigitization. Some EDCs may induce myxedema, or a shrinking of the thyroid gland (Wollman 1980). Again, myxedema can also be quantified using the methods discussed for goiter. Distinct histopathology is also associated with goiter, including a thickened capsule wall, shrinking of capillaries, fusion of the follicles, increased follicular size, and infiltration of connective tissue (partitions) entering the lobe from the capsule (Wollman 1980). The histological techniques are relatively simple, using thin sections of the thyroid gland and standard light microscopy of hemotoxylin/eosin stained tissue. Use of

thyroid morphometry and pathology, particularly in *Xenopus*, where the thyroid gland is fully visible in the intact specimen, should be included in the analysis of thyroid function.

471. Specimens for histological examination can be selectively preserved in Bouin's Solution prior to preparation. Following tissue processing, including decalcification if needed, the tissue sample can be embedded in paraffin. Microtome sectioning (4-5 μm) or step sectioning (30-32 μm between steps) can be performed prior to hemotoxylin-eosin staining. The histological examination could include changes in the gland, including hypertrophy of follicular cells, hyperplasia of thyroid follicles, size of the follicle, and degree of colloid accumulation. Use of digital photographs can be used to illustrate changes and provide a means for outside peer examination. In addition to traditional light microscopic procedures, electron microscopy (EM), particularly scanning EM (SEM) or scanning transmission EM (STEM), can be considered as a potentially useful diagnostic tool. As for light microscopy described previously, EM procedures are readily available and can be adapted for evaluation of thyroid pathology.

6.11.2.3 In vitro, Ex vivo, and In vivo Assays

6.11.2.3.1 Biochemical Markers

6.11.2.3.1.1 Corticotropin Releasing Hormone and Thyroid Stimulating Hormone

472. CRF stimulates the production and secretion of both ACTH and TSH from the pituitary. Since the structure of CRF is known (41 peptide protein), it could potential be measured, although no diagnostic test in amphibians is currently available. Either ELISA or RIA techniques could be considered.

473. TSH in humans is measure by high affinity RIA and used in combination with TH measurement to evaluate and manage thyroid dysfunction. Considering the structural similarities between mammalian and amphibian TSH, similar measurement techniques could be developed.

6.11.2.3.1.2 Thyroid Hormones

474. TH (T_3 and T_4), precursors (MIT and DIT), and deiodinase activities can be analyzed by the following methods. Since these methods have not been extensively used in amphibians, multiple methods will need to be considered. Further, biochemical measurement of thyroid activity can be measured in plasma obtained from cardiac puncture and tissue (thyroid gland or whole body). Three primary methods are available, although limited data are available on each in terms of sensitivity and reliability (Moller et al. 1983; Galton et al. 1991; Mellstrom et al. 1991; Ekins 1999; De Brabandere et al. 1998; Baiser et al. 2000). These methods include RIA, ELISA, and LC/GC-MS. Of these methods, only RIA techniques have been used to measure amphibian TH (Galton et al. 1991). Currently, ELISA and LC/GC-MS test methods have been developed for mammals, in human tests of thyroid function. In order for ELISA to be routinely used, an ELISA kit would need to be developed for amphibian TH and deiodinase. In addition, new chromatographic methods developed for human TH analysis need to be adapted for amphibian samples. At this point, conventional RIA analysis methods of TH are adequate. Regardless of method, quality assurance (QA) measures associated with ELISA and RIA analyses should include an evaluation of cross-reactivity with other hormones or similar substances, evaluation of linearity using standard curves, and the use of standard additions to assess recoveries. Perhaps the most promising of the biochemical techniques are described by Simon et al. (2002), who have recently developed a new approach for the analysis of iodinated organic species in serum and whole body tissue homogenates using liquid chromatography-inductively coupled plasma-mass spectrometry (LC-ICP-MS). This method enabled the simultaneous quantification of iodide, T_4, T_3, rT_3, MIT, DIT, as well as five additional presently unidentified iodinated molecules in *Xenopus* larvae. This extraction method will require appropriate validation before it can be used to quantitate iodocompounds in tadpole tissue.

6.11.2.3.1.3 Iodothyronine Deiodinases

475. Two deiodinase isoforms are present in most anurans. One isoform, type II, catalyzes the conversion of T_4 to T_3 in the thyroid and various target tissues, whereas the other isoform, type III, selectively inactivates T_3 and T_4 by converting them to T_2 and reverse T_3 (Huang et al. 1999). It is thought that type III deiodinase in anurans (*X. laevis*) is responsible for protecting the tissues from circulating TH. Koopdonk-Kool et al. (1993) developed a method for measuring deiodinase activity by measuring the conversion of [^{125}I]T_3 to T_2. In most cases deiodinase activity is not considered in evaluating thyroid function; however differences in tissue levels of T_4 and T_3 can in some cases be explained by differing deiodinase activities. Further work will be required to fully determine the usefulness of deiodinase measurement in evaluating thyroid dysfunction.

6.11.2.3.1.4 TH Transport Proteins

476. TH synthesis and secretion, TH transport by carrier proteins, and TH binding to TR constitutes the primary thyroid axis pathway. Thus, measurement of TH transport proteins and TR provide a measure of TH activity and responsiveness (Tata 1999). The most practicable methods of evaluating these processes appear to be quantifying changes in protein levels during metamorphosis. Conventional ELISA is suitable for the analysis of specific transport proteins, such as transthyretin (Yamauchi et al. 1993). However, such analysis requires plasma samples which are difficult to obtain from tadpoles. Evaluating TR gene expression also represents a suitable method for evaluating the up and down regulation of TR. Immunohistochemical analysis of TR in the whole organism can be used to regionally quantify the presence of TR in a metamorphosing tadpole. The success of this approach depends on procurement of robust TR antibodies which are not readily obtained. Overall, analysis at the RNA level is probably more appropriate.

6.11.2.3.1.5 Clinical Tests of Thyroid Function

477. Aside from analytically measuring pituitary hormone and TH levels, associated regulatory enzymes of the thyroid axis, and TH transport proteins, and other classical methods of measuring thyroid function in higher animals, including humans, could be considered. These classical assays include, radioactive I (^{125}I) uptake by the thyroid and T_3 resin tests, which measure thyroglobulin binding protein among a host of others (Thomson 1974). None of these tests have been specifically adapted for use in amphibians due to the advent of more sophisticated molecular techniques. Therefore, it is unlikely that the classical methods of measuring thyroid dysfunction in humans will be more effective in measuring thyroid disruption in amphibians, than the methods already described or the approaches discussed in the following section.

6.11.2.3.2 Molecular Markers of TH Action

478. Three molecular approaches for measuring TH-induced metamorphosis are currently being evaluated: single gene expression assays, multiple gene expression assay, and somatic or germinal transgenesis of relevant reporter gene constructs. The single gene expression assays reviewed included RT-PCR and ribonuclease protection assay (RPA) technology. The multiple gene expression assays reviewed include differential display and gene array techniques. Development of transgenic lines expressing novel TH-inducible gene sets (i.e., TRβ and related TREs) is possible. However, the complexity and time required to create a transgenic line makes it somewhat less attractive than the gene expression assays. The potentially most useful molecular assays are the gene array and RT-PCR approaches. Differential display is plagued by difficulty in interpretation and quantification of the results of simultaneous multiple gene expression, and will thus not be considered further. Although the RPA technology could potentially be used to monitor single gene expression, the newer RT-PCR techniques are

most efficient and sensitive. Based on this information, measurement of TH-inducible gene arrays (TRβ, TRα, ST_3, and TH-inducible genes) could be used to measure changes in multiple gene activity as the result of EDC exposure. A more simplistic method is to use RT-PCR to measure changes in single gene activity. In this case, changes in TRβ, ST_3, arginase, or other relevant TH-induced genes could be quantitatively measured for changes as the result of EDC exposure. Furthermore, both RT-PCR and gene arrays could be used as an endpoint in the short-term morphological tests, along with biochemical measurements, to determine if the responses are the result of thyroidal or non-thyroidal processes. The primary advantage of the gene array technology over the RT-PCR approach is that it is capable of monitoring the activity of multiple genes at one time. Since TH-induced metamorphosis is the result of multiple genes acting within a highly complex program, it is more realistic and potentially useful.

6.11.2.3.2.1 Single Gene Expression Assays

479. One of the most promising single gene molecular biomarker assays is RT-PCR. RT-PCR methodologies for specifically measuring TRβ gene expression changes, as the result of exposure to potential EDCs in *X. laevis* tail biopsies, were developed by Veldhoen and Helbing (2001). RT-PCR analysis of ST_3 or other relevant gene activity during prometamorphosis could also be considered. RT-PCR technology is based on the construction of cDNA from isolated RNA using reverse transcriptase. The cDNA and cDNA primer fragment (i.e., TRβ) are amplified. The amplified DNA products are then separated on an agarose gel and the amplified DNA bands are quantitatively analyzed using densitometry. RT-PCR techniques have been used to measure the induction of vitellogenin genes in *Xenopus* as the result of exposure to the weakly estrogenic compound bisphenol A (Kloas et al. 1999). Work by Veldhoen and Helbing (2001) demonstrates that quantitative analysis of single gene activity, such as TRβ, is feasible. If the TH-response genes selected for evaluation are ubiquitous, other tissues could be sampled besides the tail, including the hind limb, using a similar biopsy approach. Using PCR-based subtractive hybidization, Denver et al. (1997) isolated 34 different cDNAs for TH-regulated genes in the diencephalons of *Xenopus* tadpoles. Northern blots verified that the mRNAs were regulated by TH and were expressed during metamorphosis. Most of these genes were found to be up-regulated by TH within 4-8 h and 14 of the 34 are regulated by TH only in the brain.

480. A semiquantitative RT-PCR assay for the β-subunit mRNA of TSH (TSHβ from *X. laevis* was developed (Opitz et al. 2002) and used to study TSHβ mRNA expression in tadpoles exposed to the anti-thyroid chemical ethylenethiourea (ETU). TSHβ mRNA is exclusively expressed in the *Xenopus* pituitary, but since a single pituitary gland is too small to obtain a sufficient amount of tissue for RT-PCR, whole brains were instead used for RNA isolation. Extra-pituitary TSHβ mRNA expression has been described in other species. However, Manzon and Denver (2004) recently measured TSHβ in dissected pituitaries. The developmental expression profile of TSHβ in tadpoles during spontaneous metamorphosis was determined by means of RT-PCR analysis of total RNA extracted from whole brain homogenates. TSHβ mRNA levels increased from stage 54 to 58/59 and rapidly declined thereafter to low levels confirming the results of Buckbinder and Brown (1993). However, these results are not confirmed by analysis of pituitary TSH (Manzon and Denver 2004). In fact, Krain and Denver (2004) showed that elevated TSHβ mRNA remains elevated through the completion of metamorphosis, as does whole body T_4. The highest signal was obtained in whole brain homogenates of tadpoles at stages 58/59. The expression level in unexposed tadpoles and tadpoles treated with 10, 25 and 50 mg/L ETU for up to 5 weeks was compared (Opitz et al. 2002b). Exposure of tadpoles to 10 mg/L ETU did not affect metamorphic development whereas development was slowed by 25 mg/L ETU and became completely arrested at stage 53/54 at an ETU concentration of 50 mg/L. Expression levels of TSHβ were compared between control tadpoles (stage 58/59), tadpoles treated with 10 and 25 mg/L ETU (stage 58/59), and tadpoles treated with 50 mg/L ETU (53/54). Semiquantitative RT-PCR analysis of whole brain homogenates revealed that all ETU treatments increased TSHβ mRNA expression at least two-fold over control levels, respectively. From these results, it was concluded by Opitz et al. (2002) that TSHβ mRNA expression may provide a very sensitive biomarker

for detection of anti-thyroidal activities in *Xenopus* tadpoles. Conversely, it can be argued that a two-fold increase does not represent a sensitive biomarker. TSH expression can increase greater than 10-fold with goitrogen treatment in various species (Manzon and Denver 2004).

481. Currently, further studies are in progress addressing the stage-dependent induction of TSHβ and the temporal expression profile of TSHβ mRNA, respectively, following ETU treatment. These studies are aimed at the identification of the most sensitive developmental period for TSHβ induction in response to anti-thyroidal compounds and the minimum treatment duration until up-regulation of TSHβ becomes detectable (Opitz et al. 2002). However, studies are still needed to characterize TSHβ gene activity following treatment with modulators of monodeiodinase activities because at least D2 activity appears to play an important role in mediating feedback responses at the pituitary level (Huang et al. 2001; Schneider et al. 2001). Furthermore, a detailed characterization of pituitary and thyroidal activity in response to T_4 and T_3 exposure is warranted for a better understanding of the compensatory feedback mechanisms being activated along the pituitary-thyroid axis in response to inhibiting and stimulating compounds. Since most of the recommended endpoints for an amphibian prometamorphosis assay are related to these compensatory activities, a comprehensive evaluation of these biomarkers should be performed not only for inhibitors of TH synthesis but also for modulators of monodeiodinase activities.

482. Analysis of TRβ gene expression following short-term treatment (24 to 72 h) of stage 50/51 tadpoles with a given test substance may provide a rapid means to detect direct agonistic activities (Opitz et al. 2002). Results from a study by Veldhoen and Helbing (2001) as well as recent data from our own experiments suggest that incorporation of an acute challenge exposure with TH can enhance the utility of such an short-term molecular assay to detect chemical interaction with TH action which otherwise would not be noticed. Analysis of gene expression after an acute challenge with TH may reveal various possible modes of action for chemicals to interfere with TH action in target cells. Since TH treatment leads to a rapid increase in the cellular TR population, this may, as a secondary effect, increase the sensitivity of tissues to weak agonists. Thus, comparison of TRβ induction after acute TH exposure of untreated tadpoles and tadpoles treated with the test substance may indicate the potential of a test substance to act synergistically or as an antagonist. Further, it remains to be investigated whether inhibitors of monodeiodinase activities can affect the pattern of gene expression within an acute challenge assay. The advantage of an acute challenge exposure is that it reduces the possibility that chemical effects are obscured by compensatory activities at different levels.

6.11.2.3.2.2 Multiple Gene Expression Assays

483. A more recently developed technique for evaluating multiple gene activity involves the use of gene arrays. DNA micro- and macro- arrays are powerful tools in the analysis of differential gene expression. The cDNA micro-arrays are capable of profiling gene expression patterns of tens-of-thousands of genes in a single experiment. In this technique, DNA targets, in the form of 3' expressed sequence tags (ESTs), are arrayed onto glass slides or membranes and probed with fluorescent- or radio-labeled cDNAs (Duggan et al. 1999). TH-mediated gene expression patterns have been identified in the rat (Witzel et al.,2001) and *X. laevis* (Denver et al. 1997) using cDNA expression arrays and a slightly different technique, PCR-based subtractive hybridization. Studies using this approach have identified sets of T_3-responsive genes in various tadpole tissues, including the tail (Wang and Brown 1993; Brown et al. 1996), hind limb (Buckbinder and Brown 1992), brain (Denver et al. 1997), and intestine (Shi and Brown 1993). These analyses have demonstrated that some early TH-response genes are common to all tadpole tissues, whereas others are tissue specific. Genes identified by Denver et al. (1997) to be up-regulated during prometamorphosis and metamorphic climax include *xh*4, *xh*7, *xh*15, *xh*6, *xh*1, TRβ, and bZIP. Gene array constructs using TH-responsive genes and TREs could be developed to evaluate the effect of potential EDCs on the expression of multiple TH-dependent genes. Although the gene array technology offers

advantages over differential display, it is not yet clear how interpretable and technically feasible the gene array analysis approach will be in a screening test format.

6.11.2.3.3 Transgenesis

484. An *in vivo* approach to the study of gene function is through gene knockout and transgenic lines. To date, no gene knockouts have been developed in amphibians. However, two methods of developing transgenic lines have been established using amphibian species. The first approach involves the nuclear transplantation of somatic nuclei into an enucleated oocyte. Once the transplantation is complete, the oocyte is fertilized. Kroll and Gerhart (1994) used this approach to transfect a gene of interest into a *X. laevis* tissue culture cell line. Successfully transfected nuclei are then microinjected into newly fertilized embryos. However, this approach has not been largely successful in growing embryos beyond a young larval stage. Thus, the use of transfected somatic nuclei transgenesis is not well suited for studying metamorphosis. The inability to raise the transgenic specimen beyond early larval stages prompted Kroll and Amaya (1996) to develop the second approach that uses undifferentiated sperm cell nuclei. This approach entails the insertion of a gene or genes with appropriate promoters in a plasmid. The plasmid is then linearized using restriction enzymes. The linearized plasmid and *X. laevis* sperm cell nuclei are mixed in a high-speed extract made from *X. laevis* eggs. A short incubation period allows decondensation of the nuclei to occur, allowing plasmid incorporation into the chromatin. The transfected sperm nuclei are then microinjected into oocytes at a rate of one nucleus per egg. Although the efficiency is still rather low, the techniques compare favorably to similar approaches developed for the mouse and zebrafish.

485. The availability of many genes involved with metamorphosis in combination with tissue-specific promoters, will eventually allow construction of a transgenic line that models the expression of a series of genes important for successful metamorphosis. Adaptation to *X. (Silurana) tropicalis*, a diploid organism with a shorter lifecycle, further increases the feasibility of these studies. A transgenic line exploiting TH/TR response elements could be developed. Activation of these response elements by the action of an exogenous EDC could be marked by a marker protein. For example, Oofusa et al. (2001) evaluated the activity of the thyroid responsive element (TRE) using *X. laevis* carrying a transgene containing the 5' upstream region of the TRβ A1 gene and a green fluorescent protein (EGFP) gene. EGFP expression was then monitored throughout the entire premetamorphic, prometamorphic, and metamorphic climax periods. TRβ expression was found as early as neurula stage at low levels, with low activity during the remainder of premetamorphosis, culminating at metamorphic climax. A similar transgenic *Xenopus* line could be developed to measure TRβ expression and the influence of potential EDCs on gene expression. Wang and Brown (1993) and later Furlow and Brown (1999) identified a novel lencine zipper transcriptional factor (TH/bZIP) that is induced by TH during metamorphosis in transgenic *X. laevis*. Two genomic TH/bZIP genes regulated by an adjoining DR+4 TRE have been found in *X. laevis*. The effect of potential EDCs on up and down regulation of TH/bZIP could also be measured using an EGFP marker. Overall, in accordance with the work of Luze et al. (1998), Ulisse (1996), and Rowe et al. (2002), it is possible that a reporter gene assay created from either somatic or germinal transgenesis could be used to mark thyroid axis disruption in a quantifiable, rapid process. Further, this assay could be used in conjunction with a broader-based morphological assay.

6.11.2.3.4 Organ/Cell Culture

486. Consistent TH-induced tail resorption is relatively easy to monitor in organ cultures (Weber 1967; Tata et al. 1991). Whole tail cultures could be used to evaluate TH agonists and antagonists downstream from the thyroid gland. More specifically, tail culture assays could potentially evaluate TH interaction with the TR, TR activation of the TREs, and the cascade of molecular events associated with selective cell death in the tail. Similar transfected cell culture lines could also be established to measure the influence of EDCs on TREs and associated gene products (Denver et al. 2002).

6.11.2.3.5 Receptor and Protein Binding Assays

487. Since EDC binding to the TR and plasma TH transport proteins represents an additional process, which may be affected by EDCs, a receptor or transport protein binding assay could also be considered. Currently, TR binding assays, which measure the relative binding affinities of potential EDCs to the TR, are available and are being evaluated (Burkhart et al., personal communication). In addition, a transthyretin binding assay has also been developed for the study of TH transport (Denver et al., personal communication). However, this assay technique has not been evaluated for use in evaluating EDC activity on the thyroid axis in amphibians.

6.11.3 Candidate Anuran Assay Protocols

488. A review of candidate anuran assay protocols for measurement of thyroid disruption are summarized in the following paragraphs, and can also be found in the Detailed Review Paper on Amphibian Metamorphosis Assay for the Detection of Thyroid Active Substances [OECD Series on Testing and Assessment No. 46] (OECD 2004).

6.11.3.1 16-d Metamorphosic Climax Assay

489. The 16-day Metamorphic Climax Assay is conducted during the final period of metamorphosis, which is most prominently marked by the resorption of the tail and the development of the forelimb. As originally proposed by Fort et al. (2000), this assay primarily quantitatively evaluated the rate of tail resorption in *X. laevis*, although maturation of the skin and forelimb development were noted anecdotally. Due to higher than acceptable levels of variability in the rate of tail resorption, the assay has been modified somewhat to increase robustness. However, this variability is primarily due to the overwhelming influence of the endogenous TH peak that occurs during this developmental window. Thus, it is anticipated that this stage would be relatively insensitive to TR agonists since the system is fully stimulated by the TH cascade. Conversely, this stage may also be insensitive to TR antagonists as the endogenous TH surge initiates a cascade of gene activity that is insensitive to most synthesis inhibitors (Brown et al. 1996; Denver et al. 1997).

490. As originally described, the primary drawback to this approach, aside from questionable sensitivity, is that it relied merely on gross morphological endpoints. As previously discussed, in order to distinguish between thyroidal- and non-thyroidal-based changes in developmental rates, other biochemical and molecular endpoints need to be incorporated into the test protocol that can establish mechanistic links to the observed morphological effects. Based on this information, a modified morphological test based on the original metamorphic climax assay that incorporates a more sensitive stage of the metamorphic period, such as early prometamorphosis, that is capable of utilizing biochemical and molecular endpoints would appear to be more advantageous.

6.11.3.2 Rationale for the Use of a Prometamorphosis Model for Detecting Thyroid Dysfunction

491. As previously indicated, two primary developmental periods need to be considered in the exposure regime used in the Amphibian Metamorphosis Assays, pre/prometamorphosis (stages 51 to 54) and metamorphic climax (stages 61 to 66). A toxicant may or may not induce an effect during these periods depending on the mechanism of action. Most compounds that adversely affect the thyroid axis would show activity at least during prometamorphosis. Some of these thyroid disruptors might also demonstrate effects during metamorphic late prometamorphosis (NF stages 58-61), but effects on metamorphic climax proper (NF stages 61-66) are unlikely since metamorphic climax occurs to rapidly to be modulated by EDCs. High levels of circulating endogenous TH also reduce the sensitivity of this metamorphic period. Thus, since a short-term test is desired, an exposure protocol incorporating either

prometamorphosis or metamorphic climax would appear to be most effective. It is likely though that late premetamorphosis to early prometamorphosis (stages 51 to 58/59) will be the most sensitive period. However, it is possible that a toxic insult could result in abnormal development of the thyroid during the premetamorphic stage. The main focus of anuran thyroid disruption assay should focus on modes of action leading to perturbation of T_4 synthesis, transport and elimination enabling the identification of TH agonists or antagonists. However, detection of chemical effects on target cell action of TH must also be considered.

492. Significant T_4-secretory activity of the thyroid glands begins at stage 54 and circulating T_4 concentrations rise throughout prometamorphosis (Leloup and Buscaglia 1977). Initiating the exposure with stage 52 premetamorphic tadpoles (that is prior to the activation of thyroidal activity) may allow some time for inhibitory substances to exert their effects before endogenous production of T_4 sets in. Thus, premetamorphic exposure is suggested to increase the sensitivity of the assay towards inhibitors of thyroid gland function.

493. The precise function of feedback mechanisms along the hypothalamus-pituitary-axis in anuran larvae is not completely understood. During metamorphic development, TSH α- and β-subunit mRNA expression levels rise in parallel with increasing TH plasma levels throughout prometamorphosis, reaching their maximum levels around climax and declining thereafter to low levels (Buckbinder and Brown 1993; Okada et al. 2002). The critical role of TSH to stimulate thyroid activity during metamorphosis has been shown by immunoneutralization of endogenous TSH using antisera against mammalian TSH (Eddy and Lipner 1976) or hypophysectomy (Dodd and Dodd 1976) both leading to metamorphic retardation. Further, it has been demonstrated that amphibian TSH directly stimulates T_4 release from larval thyroid glands (MacKenzie et al. 1978; Sakai et al. 1991).

494. Importantly, it has been shown that inhibition of TH synthesis in anuran larvae by anti-thyroid compounds leads to increased mRNA expression of the α- and β-subunits of TSH (Buckbinder and Brown 1993; Huang et al. 2001), increased TSH protein synthesis and secretion in the pituitary (Goos et al. 1968; Miranda et al. 1995), hyperactivity of the thyroid gland (Goos et al. 1968; Hanaoka 1967; Miranda et al. 1996) and formation of goiter (Buckbinder and Brown 1993). Accordingly, valid measurements to detect antithyroidal activity may comprise histological analysis of thyroid and pituitary tissues, determination of T_4 and T_3 concentrations, and immunohistochemical or molecular analysis of TSH expression.

495. Background concentrations of TH are very low in premetamorphic tadpoles because significant T_4-secretory activity of the thyroid gland has not yet started. However, premetamorphic tadpoles already display a competence to respond to exogenously added TH by upregulating TH-responsive gene expression programs leading to precocious induction of morphological changes (Tata 2000). The presence of TRβ in premetamorphic tadpoles provides the basis for this competence. One gene which is upregulated by TH is TRβ (Yaoita and Brown 1990). Upregulation of TRβ occurs within a few hours after TH administration. Maximal induction is achieved within 48 hours after T_3 treatment (Kanamori and Brown 1992; Yaoita and Brown 1990). Further, induction of TRβ gene expression is unique to TH as other hormones did not show a direct effect on this gene activity (Kanamori and Brown 1992). These characteristics make TRβ one of the candidate molecular biomarkers for the study of TH-mimicking activities of environmental chemicals. The utility of TRβ as a molecular biomarker of thyroidal activity in *Xenopus* was evaluated by Opitz et al. (2002). TH-induced upregulation of TRβ mRNA in different tadpole tissues was measured by means of semi-quantitative RT-PCR. TRβ mRNA expression was increased in a dose-dependent manner after 24-h treatment with T_4 (1, 5 and 10 nM) and T_3 (1, 5 and 10 nM) in both head and tail tissues with T_3 being more potent than T_4. The magnitude of TRβ induction by T_4 and T_3 over control levels was further stage-dependent being highest in stage 50/51 tadpoles. Induction of TRβ was less pronounced in stage 54/55 tadpoles, while in stage 58/59 tadpoles, concentrations of 1 and 10 nM T_4 were ineffective in inducing a significant increase in TRβ expression over control levels for both head and tail tissues. The low background expression of TRβ at stages 50/51 allowed for a very sensitive detection of TH activity when

TRβ expression was measured by RT-PCR 24 hours after treatment with T_4 (LOEC: 1 nM) or T_3 (LOEC: < 1 nM). Although additional work is needed for validation of the amphibian prometamorphosis model for detecting thyroid disruption, additional development and validation of this model is highly recommended.

6.11.3.3 XEMA Model

496. To date, the most common approach used to assess the possible effects of environmental chemicals on anuran metamorphosis was to determine whether chemical exposure could alter the time period required until emergence of the forelimbs (Ankley et al. 1998a; Cheek et al. 1999) or for completion of metamorphosis (Allran and Karasow 2000; Bridges 2000; Britson and Threkeld 1998; Gutleb et al. 2000; Jung and Walker 1997). For *X. laevis*, the time periods from hatching to forelimb emergence and completion of metamorphosis are approximately 35-40 and 55-60 days, respectively, under optimal laboratory conditions. However, in some studies, much lower developmental rates of *Xenopus* larvae have been reported (Goleman et al. 2002; Huang and Brown 2000). These differences are most likely due to differences in rearing conditions such as food availability, temperature, and density of larvae, all of which can have profound effects on the duration of the larval period.

497. The XEMA assay is a morphological assay designed to specifically detect effects of environmental chemicals on TH-regulated metamorphic development (Opitz et al., 2005). The basic premise of this model is that possible thyroid-disrupting effects of a given test substance are manifested in *X. laevis* tadpoles as morphological alterations during metamorphosis such that compounds that inhibit TH function reduces the rate of metamorphic development whereas substances which mimic or amplify TH activity cause an acceleration of metamorphosis.

498. In XEMA model (Opitz et al., 2005), exposure was initiated with premetamorphic tadpoles (stage 48/50) and continued for a total of 28 days in a static renewal test system. During the exposure period, control tadpoles developed to late prometamorphic/early climax stages (stages 58/59) under optimized rearing conditions. The main endpoints of the initial test protocol were developmental stage and whole body length (Opitz et al. 2005).

6.11.3.4 German Ring Study

499. The utility and inter-laboratory transferability of the XEMA assay was evaluated in an informal ring study using several substances known to affect the thyroid system was performed (Opitz et al. 2005). The initial test protocol for the XEMA assay was evaluated by six laboratories. For this validation study, tadpoles were exposed to five different concentrations of amitrole (8.4, 42, 84, 210, 420 mg/L), zineb (1.37, 2.75, 13.7, 27.5, 137.5 µg/L), and ETU (5, 10, 25, 50, 100 mg/L). In addition, 75 mg/L 6-n-propyl-2-thiouracil (PTU) [inhibitory control], 1 µg/L T_4 [stimulatory control], and solvent controls were concurrently evaluated. All exposure experiments used an aqueous route of exposure. Treatment solutions were changed out completely and renewed with new test solution three times a week.

500. The use of tadpoles at early life stages was found to be a prerequisite in order to ensure a high sensitivity of the assay towards stimulatory activities. In general, a significant acceleration of metamorphosis was still detectable at a low T_4 concentration of 1 µg/L. Treatment of tadpoles with PTU produced the predicted inhibitory effect. PTU completely inhibited progression of metamorphosis beyond stage 54. Consistent with observations that amitrole and ETU inhibit TH synthesis in mammals, dose-dependent inhibition of metamorphosis was observed for both compounds in the XEMA assay whereas zineb showed no effects on development but produced toxic effects at the highest concentrations used (137.5 µg/L). The endpoints observed were developmental stage and whole body length.

501. Using the primary endpoint developmental stage, the most sensitive time point to detect inhibitory effects of ETU and amitrole was day 28. Inhibitory effects of the highest test concentrations of both compounds, which produced complete inhibition of metamorphosis, were already evident at earlier time points during the test phase (days 14 and 21). Lower concentrations of these inhibitors producing only incomplete inhibition of TH synthesis required a longer time to produce a significant effect on development. Generally, modest inhibitory activities became not apparent as retardation in hind limb development throughout early and mid-prometamorphosis (days 14 and 21). However, concurrent with the increasing demand on TH for development through late prometamorphic stages 57/58, delays in metamorphic development caused by weaker inhibitory activities could be detected at day 28. From these data, the XEMA assay appears to provide a relatively sensitive and viable approach to assess the biological effects resulting from thyroid disruption in *Xenopus* tadpoles. However, the necessity of a 28-d assay and the practicability of a short-term test format targeting later premetamorphosis and prometamorphosis is currently being evaluated. The OECD Amphibian Expert Group concluded that the 28-d XEMA assay was unnecessarily prolonged. Modified assays beginning with stage 51 for 21 days or stage 54 for 14 days were recommended for concerted evaluation.

6.11.3.5 Partial Pre-/Prometamorphosis Assays

502. As recommended by the OECD expert group, an approximately 21-day pre-/prometamorphosis assay protocol exposing *X. laevis* from late premetamorphosis stage 51 or a 14-d assay exposing larvae from stage 54 could potentially be the most advantageous whole organism exposure design. In this scenario, the rate of development as measured by developmental stage, growth (whole body length and weight), hind limb length and status of differentiation, and thyroid gland histology is measured. Biochemical measures of TH (whole body, thyroid gland and plasma) and possibly a molecular screen of TH-inducible gene expression (i.e., TRβ) can also be included. Although tail resorption was used as a morphological marker of metamorphosis in this study, it is not unreasonable that hind limb development would follow the same pattern, only with increased sensitivity. The OECD Phase 1 validation study involved many groups, and compared the responsiveness and sensitivity between a 14-d exposure using stage 54 specimens and a 21-d exposure using stage 51 organisms to determine which exposure format would be preferable using the reference compounds T_4 and PTU (FEL/Battelle 2005). US labs tested additional chemicals, including methimazole, dexamethasome, phenobarbital, and pregenalone-16α-carbonitrile (PCN). Results from these studies indicated that although the 21-d exposure regime was slightly more sensitive, the 14-d protocol performed well in terms of anticipated response. Endpoints evaluated included developmental stage, whole body weight, and thyroid gland histology.

6.11.3.5.1 OECD Phase 1 Validation Trial

[The following information is quoted from the Phase 1 report.]

503. "The first OECD ad hoc Expert Meeting on Amphibian Testing (June 26 – 27, 2003, Duluth, Minnesota, USA) reviewed and discussed existing testing approaches and protocols and agreed on an action plan for Phase 1 validation work (OECD 2003). The two main outcomes of this Expert Meeting were that (I) X. laevis represents the primary candidate for a test species to be used in the Amphibian Metamorphosis Assay, (II) an exposure phase covering pre and prometamorphic development but not metamorphic climax offers considerable potential for the development of a sensitive test protocol.

504. "Experience from previous studies conducted in various laboratories indicated that a test protocol which includes exposure of tadpoles from late premetamorphic stages (e.g., stage 51) throughout late prometamorphic stages 58/59 would require a test duration of approximately 21 days. However, the Expert group also acknowledged the need for a short exposure duration due to the intended use of the assay for

screening purposes. Therefore, an alternative 14-d test protocol was proposed involving exposure of tadpoles from early prometamorphic stage 54 throughout late prometamorphic stages 58/59.

505. "Accordingly, the primary objective of validation Phase 1 was a comparative evaluation of the utility and sensitivity of the two proposed exposure scenarios to detect stimulating and inhibiting effects of thyroid system-disrupting substances on X. laevis metamorphosis. For this purpose, exposures were initiated with X. laevis tadpoles at developmental stages 51 and 54, respectively. Exposure of stage 51 tadpoles was continued for a total of 21 days and exposure of stage 54 tadpoles was continued for a total of 14 days. Tadpoles were exposed to 4 different concentrations of the test substance (n= 2 replicates per concentration) and a dilution water control group (n= 2 replicates). All exposure experiments used an aqueous route of exposure. The chemicals included in this testing were 6-propylthiouracil (PTU) and thyroxine (T4). PTU is a well studied chemical known to inhibit thyroid hormone synthesis and T4 is the native prohormone. Test concentrations for both compounds were selected based on the experience of the three participating laboratories in conducting related work with X. laevis.

506. "Participants of the first OECD ad hoc Expert meeting further agreed on a set of morphological, histological and molecular biological endpoints that should be evaluated during Phase 1 work with regard to their relevance, sensitivity and diagnostic value for detection of thyroid system related effects caused by the test chemicals. In addition, efforts towards the standardization of endpoint measurements were regarded as another major objective of the Phase 1 validation studies."

6.11.3.5.2 OECD Phase 2 Validation Trial

507. An inter-laboratory validation study of a 21-d *X. laevis* prometamorphosis exposure format developed by the OECD ad hoc Amphibian Expert Group based on the Phase 1 results is currently being performed by six independent laboratories using three test materials: perchlorate, iopanoic acid (IOP), and T_4. Endpoints included in the study are: developmental stage, whole body weight, snout-vent length, hind limb length, and thyroid gland histology. Results from these studies are intended to validate the recommended amphibian metamorphosis assay protocol.

6.11.4 Recommended Assay Protocols

508. A detailed review of recommended anuran assay protocols for measurement of thyroid disruption can be found in OECD DRP No. 46, Detailed Review Paper on Amphibian Metamorphosis Assay for the Detection of Thyroid Active Substances (OECD 2004). An amphibian metamorphosis assay initiated at NF stage 51 and conducted for 21 days using developmental stage, hind limb length, SVL, whole body weight, and thyroid histology will form the primary core test. Biochemical measurement of THs, and possibly molecular markers, such as TRβ gene expression, will be considered.

6.11.4.1 Species Selection Criteria

509. Two objectives in selecting an amphibian species are to select species amenable to short-term screening assays for measurement of thyroid disruption, and longer term assays that include advanced developmental and reproductive endpoints. The minimal attributes of a test species are described in the following eight criteria:

1. The species must be amenable to continuous culture in the laboratory;

2. Reproduction in the laboratory must be routine throughout the year, using either naturally occurring reproduction or some type of hormonal induction;

3. Larvae must be able to be routinely reared to predetermined developmental stages;

4. The developmental rate for the interval included in any test must be relatively fast so that the effects are observed quickly, thus minimizing test duration and test costs;

5. The endpoints which constitute the test data that will be used for regulatory or pre-regulatory action need to be supported by a sufficient knowledge base that indicates that they are relevant to the question at hand.

510. In addition to the minimal criteria noted above, it would be highly beneficial for the subject species to have additional information relevant to the following areas:

1. Genetic information, including gene sequences of thyroid axis-related genes and some knowledge of the genetic programs associated with TH dependent processes;

2. Biochemical information on the endocrine axis, particularly of the hypothalamus-pituitary-thyroid (HPT) axis; and

3. Metabolism information, especially as it relates to TH homeostasis.

6.11.4.2 Test Species

511. The only anuran species which meets the minimal criteria established above is *X. laevis*. This species is routinely cultured in laboratories worldwide and is easily obtainable through commercial suppliers. Reproduction can be easily induced in this species throughout the year using hCG injections and the resultant larvae can be routinely reared to selected developmental stages in large numbers to permit the use of stage-specific test protocols. The developmental rate for *X. laevis* is relatively rapid compared to the two ranid species commonly used in biological and toxicological research, *R. pipiens* and *R. catesbeiana*. In terms of thyroid dependent post-embryonic development, more is known about *X. laevis* than any other anuran species. In addition to meeting the minimal requirements stated above, the genetic information regarding *X. laevis* is more extensive than other anurans, especially in the area of the thyroid axis, where numerous publications have detailed the genes and the genetic program involved in the process of metamorphosis. Finally, the information on the biochemical and metabolic control of TH in this species is well-developed, and includes information on all of the typical HPT modulators as well as peripheral tissue enzymes, such as the deiodinases, which ultimately control the local and downstream effects of TH.

512. The only alternative species that should be considered is *X.* (*Silurana*) *Tropicalis*. This species is similar in terms of ease of culture and reproduction. The primary advantages of this species are: 1) relatively rapid developmental rate that could shorten test protocols, especially those that include reproductive endpoints (not specifically discussed in this DRP), and 2) the genome of this species is diploid which will eventually facilitate the development and use of molecular endpoints. However, at this time, there is too little experience in the broader scientific community to support the selection of this species as the primary species for a Tier I screen. In addition, disease susceptibility may be greater in *X.* (*Silurana*) *Tropicalis* than in *X. laevis*, making them more difficult to rear successfully in the laboratory. Eventually, as the genetic information is developed for this species (major initiatives have been proposed to sequence the genome of *X.* (*Silurana*) *Tropicalis)* and as more laboratories develop biological and toxicological information to support the use of *X.* (*Silurana*) *Tropicalis*, this species may replace *X. laevis*.

513. In terms of a Tier II test that includes advanced developmental (e.g., gonadal development) and reproductive endpoints, *X.* (*Silurana*) *Tropicalis* is superior to *X. laevis*. The primary advantage is that sexual maturity occurs in about 4 to 5 months in *X.* (*Silurana*) *Tropicalis*, whereas *X. laevis* requires 1.5 to 2 years. One possible problem common to both species is that spontaneous reproduction cannot be reproducibly achieved in the laboratory and amplexus must be induced by hCG injections. However,

spontaneous reproduction of other amphibian species in the laboratory also does not generally occur and breeding in the laboratory is substantially more complex.

6.11.4.3 Description of Method

514. The utility of any biological protocol is necessarily constrained by inherent limitations of the model, costs of conducting the protocol, variability inherent in the endpoint responses, specificity of the response with respect to the hypothesis, and sensitivity of the endpoints using a practical and reproducible approach. Given these general considerations, it is important to focus the development of a Tier I screen for thyroid disruption on a specific hypothesis. This hypothesis is, "*exposure to a test chemical causes changes in the homeostasis and action of thyroid hormone on the thyroid axis*."

515. In addition to the basic hypothesis, it should be the goal of this approach to develop a protocol which serves as a generalized vertebrate model that can be used to help evaluate the risk concerns among vertebrates. In order to accomplish this goal, it is necessary to understand the mechanisms involved in thyroid perturbation. Extrapolation to other species is dependent on defining the similarities and dissimilarities at the mechanistic level. There are many conserved aspects of the thyroid pathway among vertebrates, especially in the HPT axis. For example, substantial homology exists for many of the genes associated with TH homeostasis. And, in practice, commonly used inhibitors of thyroid synthesis behave similarly in anurans as they do in mammals. In combination, these facts support the plausibility that results obtained from a *X. laevis* model could be extrapolated to other vertebrates.

6.11.4.4 Specific Protocol Recommendations

6.11.4.4.1 Developmental Stage

516. The general developmental period upon which an assay should be developed should focus on late pre- and prometamorphosis. The rationale for this is that 1) the thyroid system is just becoming fully functional, 2) tissues are competent to respond, and 3) the pre-/prometamorphosis period is more sensitive to inhibition and stimulation than climax. An additional argument that has merit is that the transition period from pre- to prometamorphosis would be particularly sensitive to perturbation of the TH pathway because the exposures would be initiated with organisms that are initially naive to TH, but that TH synthesis would be developmentally acquired during the protocol. Effects of inhibition that precede endogenous production of TH could be enhanced because there is no TH pool to promote TH-dependent development in the absence of nascent synthesis. Conversely, effects of stimulation could be enhanced since there is no TH at the initial stages of the study. The Phase 1 OECD inter-laboratory study (Opitz et al. 2005) and the outcome of the Phase 2 OECD ring study will provide valuable insight into the specific stages utilized for the screening of thyroid disruption. Results to date (Opitz et al. 2005) suggest that the premetamorphic stage 51 larvae would be an effective initiation point and that 21-d of exposure should be sufficient to evaluate the effects of thyroid axis pertubation. Alternatively, an exposure format initiated at prometamorphic stage 54 conducted for 14-d should also be considered.

6.11.4.4.2 Endpoints

517. Four classes of potentially useful endpoints include: 1) apical morphology, including developmental stage, 2) thyroid gland histology, 3) biochemical analysis (hormones), and 4) molecular (gene expression). A detailed discussion of the use of each class of endpoints in an amphibian metamorphosis assay was provided in section 6.11.2, Relevant Endpoints in Anurans.

6.11.4.4.3 Exposure Protocol

518. The currently recommended protocol involves a 21-day pre-/prometamorphosis exposure of *X. laevis*, initiated at stage 51. Static-renewal or flow-through exposure, with adequate test substance analysis based on the physicochemical properties of the test substance, is recommended. On at least day 7 and 21 (conclusion of exposure, specimens should be digitally photographed to document morphological development and stage. Hind limb and snout-vent lengths can be measured using a scanning digitizer and total body weight measured. Specimens should be randomly selected for histological examination of the thyroid and samples should be collected for biochemical analysis (TH analysis in whole bodies, thyroid gland, and serum). Analysis of TH-induced gene expression via constructed DNA arrays is warranted, but not ready for inclusion at this point in time. More research will be required to survey this issue and will be addressed in the Data Gaps section of the DRP (Section 6.11.5).

6.11.4.4.4 Interpretation of Results

519. Results from the pre-/prometamorphic assay will be classified as: 1) histological, 2) biochemical, 3) molecular, and 4) morphological. Changes in thyroid histology, including glandular hypertrophy/atrophy, follicular cell hypertrophy, and follicular cell hyperplasia will be reviewed, with additional criteria including follicular lumen area, and colloid quality. Since histological changes can be somewhat subjective, clear histological criteria are being developed for more comparable analyses between different labs. In January 2006 the US EPA held a meeting with OECD and EPA pathologists to develop diagnostic criteria for the amphibian thyroid gland for the measures listed above. The group came up with severity grading for each criteria, and the US EPA is putting together a digital atlas of reference images for each severity grade. Chemicals that disrupt TH synthesis (goitrogens) cause hypertrophy of the thyroid due to increased stimulation from greater quantities of TSH induced by by the lack of TH negative feedback. In this case, distinct histopathology of the thyroid exists and marked reduction in circulating and tissue TH are observed. These scenarios confound interpretation of the results. Changes in TH levels directly indicate changes in thyroid activity. However, measurement of biochemical parameters alone will not demonstrate a specific histological or morphological effect. Thus, biochemical analyses combined with evaluation of apical morphology (i.e., stage and hind limb morphology) will be important in detecting a broad spectrum of thyroid disruption mechanisms. Changes in differential gene expression for arrays under control of a TRE suggest that the test substance is capable of altering TH-inducible gene activity. For example, Veldhoen and Helbing (2001) demonstrated that acetochlor positively modulated TH-induced TRβ gene expression in tail fin biopsies. Changes in downstream gene activity could potentially affect thyroid histology or TH levels, although more work will be required to specifically evaluate these relationships. It should also be noted that these TH-responsive genes can be regulated by other factors. Thus, induction or repression may or may not be related to thyroid disruption. Further study will be required to confirm a thyroid-dependent mechanism of action is responsible for the effects measured. Further, alteration in gene array activities are typically the result of biologically available TH irregardless of the mechanism of action of the thyroid disruptor. Apical morphological evaluation should not necessarily be used alone to determine if a substance alters thyroid homeostasis. Thus, if used, morphological endpoints may require other biochemical and/or molecular endpoints to distinguish between thyroid- and non-thyroid-mediated responses. However, molecular endpoints, while promising, are still being developed and are not included in the proposed assay at this time. In summary, because of the complexity in evaluating and interpreting results from these studies, use of each endpoint that has been sufficiently developed should be included until a sufficient database is developed that aids in interpretation of the data and the selection of specific endpoints to be used in the future. Based on research to date, developmental stage and hind limb development (differentiation and length), growth (weight); and thyroid gland histology have data sets to support their inclusion as endpoints in a routine screening assay. Biochemical measures of plasma and thyroid gland TH are currently being evaluated.

6.11.5 Data Gaps

520. The primary objective in identifying data gaps is to prioritize and apply resources to areas of uncertainty so as to reduce this uncertainty through research. As the EDSP process moves closer to implementation of this assay, several critical questions must be addressed. First, what responses, both organismal and sub-organismal, to established thyroid agonists and antagonists are currently known? The effects of thyroid agonists and antagonists on apical morphological changes during anuran metamorphosis are reasonably well understood. However, the relationship between changes in thyroid axis homeostasis and apical morphological changes are not as obvious. Before the effects of unknown chemicals on thyroid function can be assessed, the response of known thyroid disruptors in the recommended model system must be identified.

521. Second, which of the proposed endpoints will provide confidence that the observed effects are due to thyroid-based mechanisms? At this point, it is understood that disruption of apical morphological changes during metamorphosis may or may not be the direct result of alteration of thyroid function. It is obvious that more basic research is needed to identify and develop the perfect assay including the most valuable endpoints. More work will be required to assess confidence in the histology, biochemical, and molecular endpoints in terms of predictability of thyroid impairment.

522. Third, what is the time course of these responses? As research continues in these previously discussed areas, it will also be important to determine the time frame required to observe the effects of EDC exposure based on the selected endpoints. For example, molecular and biochemical changes may be observed more rapidly than histological or morphological changes. However, the estimated time course in which one would expect to observe these responses and changes in sensitivity relative to the time course, need to be elucidated. It should also be noted that the molecular endpoints and measures need to be developed further and linked to their downstream effects, and biochemical endpoints are still in need of some improvements to the available reagents.

523. Fourth, what is the sensitivity of the measurement endpoints? The relative sensitivities of each endpoint recommended can be estimated at this point. However, information on the specific sensitivities of each endpoint needs to be determined.

524. Fifth, when does a molecular change constitute a valid indication of thyroid perturbation? To understand with confidence at what point molecular changes are indicators of thyroid disruption, the results must somehow be shown to be related to an upstream or downstream response within the thyroid axis. If the effect is due to disruption of TH synthesis, then rescue/replacement experiments with thyroid hormone could be performed. If the effect is due to disruption of thyroid hormone transport (a likely target based on studies in mammals), then whether the compound competes for binding to transthyretin could be evaluated. If the effect is due to disruption of thyroid hormone binding to its receptor, then demonstrations with the appropriate binding and *in vitro* transfection assays are plausible. If molecular changes, such as inhibition of TRβ mRNA synthesis, can be linked to a histological, biochemical, or possibly even an apical morphological change in metamorphic processes, this relationship can be addressed. Work will be required to determine the threshold of molecular change that results in a physiological change in thyroid status. However, once this relationship and set-point are determined, the molecular assays, like gene arrays and RT-PCR techniques, will be extremely valuable.

525. Finally, and most importantly, what is the dynamic range of thyroid axis homeostasis and its relationship to gross morphological, molecular, biochemical, and histological changes? The degree to which thyroid homeostasis can be changed without adversely affecting the organism needs to be determined. In addition, the relationship between the sensitivity of thyroid axis homeostasis and the

measurements also requires understanding. To bridge these data gaps, further studies during prevalidation will need to be specifically directed toward these areas.

526. Table 6-2 shows existing or potential assays for amphibians. Table 6-3 shows points of thyroid disruption in amphibians. Specific points of thyroid disruption are listed in the left column, coupled to listings of endpoints by which it is characterized, the ultimate effect of disruption of this mechanism, whether assays are available to detect this point of disruption, and the status of this assay. These data are derived from work focused on anuran species.

Table 6-2 Existing or Potential *Ex vivo* and *In vitro* Assays - Frogs

Assay Name	Species	Major Thyroid-Related Endpoints	Target Effects Relevant to the Thyroid System	Status of the Study	
				Advantages	Disadvantages
16-18 day Metamorphic Climax Assay	Xenopus laevis	Tail resorption; T_4/T_3 levels; forelimb emergence.	Delayed tail resorption and forelimb emergence.	Straightforward metamorphical endpoint.	Lack of sensitivity relative to prometamorphosis assay; relevance to other taxa, especially mammals, is unknown.
14-day Frog Pre-/Prometamorphosis Assay (initiation at NF stage 54-58)	*Xenopus laevis*	Hind limb differentiation; T_4/T_3 levels; monodeiodinase activity; thyroid gland histology; whole body length; developmental stage.	Normal, delayed, or accelerated morphogenesis from tadpole to frog.	More sensitive than tail resorption alone; more comprehensive than other Tier I screens for thyroid; relatively short; can accommodate other biochemical and molecular biomarkers.	Relevance to other taxa, especially mammals, is unknown.
21-day Frog Premetamorphosis Assay (initiation at NF stage 51-58)	*Xenopus laevis*	Hind limb differentiation; T_4/T_3 levels; monodeiodinase activity; thyroid gland histology; whole body length; developmental stage.	Normal, delayed, or accelerated morphogenesis from tadpole to frog.	More sensitive than tail resorption alone; more comprehensive than other Tier I screens for thyroid; relatively short; can accommodate other biochemical and molecular biomarkers.	Relevance to other taxa, especially mammals, is unknown.
Xenopus Gene Expression Assay (NF stage 50/51)	*Xenopus laevis*	TRβ mRNA expression (24-h).	Induction or repression of TRβ mRNA expression.	Potentially sensitive; molecular biomarker.	Solitary endpoint; relevance to morphological effects is unknown; relevance to other taxa, especially mammals, is unknown.

Table 6-3 Points of Thyroid Disruption in Frogs

Disruption or Evaluation Site	Endpoints of Interest	Target Effects of Disruption	Assay Availability	Status of the Assay
Organismal thyroid status: circulating hormones	Serum and tissue T_4	Thyroid status affecting all target organs/tissues downstream of gland.	Yes	RIAs and ELISAs; in common use.
HPT axis activation or inactivation	Serum and tissue TSH and thyroid gland histology	Reflects HPT axis feedback effects from altered serum/plasma T_4 and T_3 due to alterations in thyroid gland function or hormone turnover. Also reflects stress input (corticosterone [positive]) and other interactive hormones (prolactin [negative]).	Yes	RIA available. New literature now available on thyroid histology.
Thyroid gland: iodide uptake	Na-I symporter and thyroid gland histology	Decreased thyroid gland uptake of iodide resulting in decreased hormone synthesis.	No	Radioiodide uptake assay not adequately developed or widely used. Symporter expression assay possible.
Thyroid gland hormone content	Thyroidal T_4 and thyroid histology	Altered thyroid hormone stores. Sensitive to release of stored hormones to maintain serum/plasma concentrations when hormone synthesis or turnover is decreased.	Yes	Difficult to isolate and sample thyroid in prometamorphic larvae.
Thyroid gland: TPO	TPO and thyroid histology	Altered thyroid peroxidase activity; effects on thyroid hormone synthesis.	No	Has not been used or validated for amphibian thyroid status evaluation.
Hormone transport: transthyretin	Tissue TH transport	Transport of TH to target tissues/liver.	Yes	Gene expression assay has been developed, but has not been properly evaluated.
Target tissues: receptor expression/binding	T_3 receptors (TR)	Altered tissue response to TH. Unknown significance.	Yes	Receptor binding assays and TRβ expression assays are available.
Target tissues: development	Developmental stage kinetics, body weight, tissue differentiation	Altered metamorphosis. Effects on rate of development, body weight for a given stage of development, state of differentiation.	Yes	Techniques have been developed and are currently being used to evaluate thyroid disruption.
Thyroid hormone excretion	Phase II liver transformation enzymes; UDP-GT	Increased T_4 excretion resulting from contaminant induction of uridine diphosphate glucuronosyltransferase activity, i.e. increase in T_4 glucuronidation which enhances excretion in bile.	Yes	Assays not validated for evaluating contaminant effects, sensitivity unknown.

7.0 THE HPT AXIS IN BIRDS AND ITS ROLE IN BIRD DEVELOPMENT AND REPRODUCTION

7.1 Overview of the HPT Axis

527. In birds, the functions of the HPT axis appear to be similar to those of vertebrates in general. Thus, there is evidence for: pituitary control being the predominant factor in regulation of thyroid gland function, both stimulatory and inhibitory hypothalamic regulation of the HPT axis, and negative feedback influences on hypothalamic-pituitary function.

7.1.1 Neuroendocrine Control of Thyroid Function

7.1.1.1 Hypothalamic Control of the Pituitary-Thyroid Axis

528. As in mammals, thyrotropin releasing hormone (TRH), which is stimulatory to the anterior pituitary production and release of thyrotropin (TSH), is present in the avian hypothalamus (Jackson and Reichlin 1974; Thommes et al. 1985) and is the same tripeptide found in mammals (Scanes 2000). The chicken TRH receptor has been cloned and shown to be very similar to mammalian TRH receptors (Sun et al. 1998). Synthetic TRH stimulates the release of TSH that can be detected by bioassay (Scanes 1974), and this release of TSH results in increases in plasma T_4 concentrations in chickens (Klandorf et al. 1978). Somatostatin also is produced in the avian hypothalamus and its brain distribution and concentration have been mapped (Geris et al. 2000). The administration of exogenous somatostatin decreases plasma T_4, presumably by depressing TSH release (Lam et al. 1986). In addition, corticotropin releasing hormone can stimulate TSH release (Meeuwis et al. 1989). In general, hypothalamic and pituitary control of thyroid function in birds appears to be very similar to that in mammals (reviews, Decuypere and Kühn 1988; Scanes 2000).

7.1.1.2 Pituitary Control of Thyroid Gland Function

529. TSH is produced in the avian pars distalis and, as in mammals, has a unique β chain and an α chain that is identical to that of the other two glycoprotein hormones, luteinizing hormone (LH) and follicle stimulating hormone (FSH). Historically, many studies have shown that both mammalian TSHs and avian pituitary homogenates stimulate thyroid gland activities (radioiodine uptake, thyroid hormone release, and gland growth; see for example work by MacKenzie 1981) and these activities are mediated via TSH receptors (Hull et al. 1995).

530. Avian TSH is not readily measured. As there are no antibodies specific to avian TSH (i.e., no β chain specific antibodies) RIAs for TSH are not available. For this reason, most studies of avian TSH alterations in pituitaries have been made by bioassay using thyroid gland endpoints such as increased T_4 release, increases in thyroidal radioiodine uptake, or indirect information about changes in circulating thyroid hormones (reviews, McNabb 2000; Scanes 2000). A subtractive RIA strategy has been used by Kühn's laboratory: TSH = total pituitary glycoprotein hormone immunoreactivity to the α pituitary glycoprotein chain (common to TSH, LH and FSH) minus LH-β and FSH-β immunoreactivity (method of Berghman et al. 1993). There is good evidence that this measurement strategy is effective for measuring distinct changes in TSH. However, one of the problems with such a subtractive framework is that all variability in the multiple measurements is attributed to the TSH fraction. Heterologous TSH antibodies (to mammalian TSH) have been used in some immunocytochemical investigations of avian pituitary development (Sharp et al. 1979; Thommes et al. 1983) and in a study of feeding effects on TSH in Japanese quail (Almeida and Thomas 1981). However, our attempts to use RIAs with heterologous TSH

antibodies for measuring avian plasma TSH showed insufficient binding to be of use (unpublished, McNabb laboratory). A new bioassay uses the cyclic adenosine 3',5'-monophosphate response of a line of cultured mammalian thyroid cells (FTRL cells) for measuring avian TSH in pituitary homogenates (Iwasawa et al. 1998). Although this technique is effective for measuring TSH in pituitaries, it appears unlikely to be sensitive enough to measure plasma TSH. To date most of the methods are not sensitive enough to measure TSH changes in plasma in many experimental contexts. The gene sequence for Japanese quail TSH has now been published (Kato et al. 1997; Catena et al. 2003) opening the way to synthesis of peptides that can be used in the preparation of avian TSH β chain-specific antibodies. Measurement of chicken TSHβ mRNA throughout embryonic and early posthatch development has verified the role TSH plays in stimulating thyroid function at different stages (Gregory et al. 1998).

531. Despite the limitations in measuring TSH described above, there is considerable knowledge about TSH effects on thyroid function in birds. Mammalian TSHs are effective in stimulating thyroid gland function in birds (McNabb et al. 1985a,b) and studies using goitrogens and thyroidectomy have been used to alter TSH release and to follow the consequent effects on thyroid gland function. In general, the effects of TSH on the avian thyroid gland are very similar to those in mammals (review Scanes 2000).

532. Pituitary control of the thyroid in precocial chicken embryos is established by the middle (day 11.5) of the 21-day incubation period although hormones are present in the axis much earlier (TRH at day 4.5 and TSH at day 6.5; Thommes et al. 1983; 1985). Establishment of axis control is followed by steady increases in circulating T_4 concentrations during the remainder of the incubation period. In altricial ring doves, establishment of HPT control occurs after hatch; thyroid function is insensitive to TSH until day 2 posthatch (McNichols and McNabb 1987) and circulating thyroid hormones remain very low through the perihatch period and then increase gradually during the first three weeks of posthatch life (see 7.1.4.1 below).

7.1.2 Thyroid Gland Function

7.1.2.1 The TSH Receptor

533. Many studies suggest that TSH control of thyroid gland function is very similar in birds to that in mammals (section 7.1.2). There do not appear to be published studies characterizing the avian TSH receptor.

7.1.2.2 Biosynthesis and Iodination of Thyroglobulin

534. It is presumed that the processes involved in iodination of thyroglobulin and thyroid hormone formation in birds correspond to those in mammals but there have been few studies that directly address this topic. Measurements of the iodide content of chickens, ducks and pigeons indicate these avian species have 2-4 X the iodide content present in rat thyroid glands (Astier 1980). This plays a role in the observation that thyroidal iodide content in adult chickens and quail is essentially unaffected by a wide range of iodide availability except when iodide is very low (Newcomer 1978; Astier 1980; McNabb et al. 1985a,b). Studies of phylogenetic differences in thyroglobulins have indicated some differences between vertebrate classes. Among the differences that may be important, the proportion of tyrosine residues (precursors to hormone formation) is ~1.6 X higher in chickens than in rats and the degree of iodination of avian thyroglobulin (1.4-2.6%) is greater than in other vertebrates (0.5-0.85%; review by Astier 1980). However, more iodine atoms in thyroglobulin appear to be "required" per molecule of hormone formed in birds than in mammals (10 iodines per T_4 in mammals, 30 per T_4 in birds; Daugeras et al. 1976). It would appear that the higher iodine content of avian thyroid glands should compensate for this lower "efficiency" in hormone formation. The physiological significance of these differences in iodination and its relationship to hormone formation between birds and mammals are not clear.

7.1.2.3 Thyroglobulin Storage in Colloid

535. The thyroid glands of birds, like those of all vertebrates, store thyroid hormones within thyroglobulin in the colloid space of thyroid follicles. The histology and ultrastructure of thyroid gland activity, with respect to follicle cell height and colloid space in relation to cell height in birds, is like that of other vertebrates (Fujita 1984; French and Hodges 1977). Much of the information on hormone storage in avian thyroid glands was developed prior to the availability of radioimmunoassays for the specific detection of thyroid hormones. Such studies of hormone storage usually separated gland hormones by chromatographic methods then determined the hormone content of different fractions indirectly by measuring their iodine content. These studies indicated that T_4 predominated in the avian thyroid, with little or no detectable T_3 present. They also indicated that in adult chickens and quail, thyroidal iodide and hormone contents appeared to be essentially unaffected by a wide range of iodide availability (review, Astier 1980). More recently, thyroidal hormone content has been measured by digesting thyroids with a bacterial pronase, extracting the hormones in ethanol and measuring thyroid hormones in the extract by RIA (method of McNabb and Cheng 1985). This technique also has shown that adult Japanese quail maintain relatively constant thyroidal hormone content with a wide range of dietary iodine intakes. In contrast, in embryos or chicks thyroidal hormone content is directly proportional to increased egg or dietary iodine availability (McNabb et al. 1985a, b). Comparisons of adult ring doves and Japanese quail with similar body size indicate that thyroidal hormone content is similar in these species with T_4 comprising at least 97% of the thyroidal hormone content in birds on an iodine-sufficient diet (McNichols and McNabb 1987). On very low iodine diets (<100µg I/kg feed) total thyroidal hormone content was markedly decreased (to 1/4 - 1/2 that on iodine sufficient diets) and the T_3/T_4 ratio in the thyroid was increased (by 2-3X).

536. The ontogenic pattern of thyroidal hormone content has been compared in precocial Japanese quail and altricial ring doves (see 7.1.4.1 for general information about these developmental patterns). In quail, thyroid hormone content is extremely low at mid incubation, increases markedly during late incubation to peak during the perihatch period, decreases to about 25% of the peak levels at 1 1/2 - 2 weeks of age, then gradually increases to adult levels. In contrast, in altricial doves, thyroidal hormone content is extremely low until after the perihatch period, rises slightly in the first week posthatch, then rises rapidly between 1 and 3 weeks of age to reach levels at fledgling that are about double those in adults. During mid to late incubation in both species, the thyroidal T_3/T_4 ratio is higher (T_3 is about 8% of gland hormone content) than in chicks or adults of both species (McNabb et al. 1985b; McNichols and McNabb 1988). The elevated T_3/T_4 ratio during embryonic life may be the result of relatively low thyroidal iodine content during development. That thyroidal T_3/T_4 ratio responds to low iodine has been demonstrated; thyroidal T_4 content is decreased and the T_3/T_4 ratio is increased in quail embryos from eggs of iodine-deficient hens compared to controls (Stallard and McNabb 1990). Recently measurements of thyroidal hormone content have been used in studies of the effects of perchlorate on avian thyroid function (see 7.6.2.3).

7.1.3 Thyroid Hormone Secretion

537. Prior to the development of RIAs for measuring thyroid hormones, a number of studies measured the daily thyroid hormone secretion rate (TSR) of T_4 by several indirect methods (reviews Astier 1980; Wentworth and Ringer 1986). Several of these methods indicated highly variable TSRs from different studies and different techniques but the values (range from 1.2 to 2.3 µg T_4/100g body weight in four studies of chickens <9 weeks of age) were generally in the range of the TSR in rats measured by the same techniques (~2 µg T_4/100g body weight) and in humans (~1.3 µg/100 g body weight; Chopra and Sabatino 2000). Many aspects of the early techniques used for avian TSR measurements are now in question because they may seriously over or underestimate TSR, so this historical work is difficult to evaluate. These studies did show that TSR is dynamic, for example, adult chickens maintained in cold temperatures had approximately double the TSR of birds maintained at warm temperatures, iodine deficient diets

lowered the TSR, and by 13 weeks of age the TSR had decreased to 0.6 µg T_4/100 g body weight (Wentworth and Ringer 1986). There do not appear to be any more recent investigations of TSR in birds.

7.1.4 Regulation of Circulating Thyroid Hormone

7.1.4.1 Ontogenic Patterns

538. In birds, as in mammals, there are two developmental modes, precocial and altricial. In brief, birds with a precocial developmental pattern are hatched at a relatively advanced stage of development characterized by functionally mature sensory systems (i.e., eyes open), capability for locomotion (i.e., relatively mature musculoskeletal function), behavioral patterns that allow some independence with relatively little parental care (e.g., imprinting to the adults) and the capability for initiating thermoregulatory responses to cooling from the time of hatching onward. In contrast, altricial young are hatched at an earlier stage of development characterized by functionally immature sensory systems (i.e., eyes closed), poorly developed musculoskeletal systems incapable of coordinated locomotion (young are nest-bound), behavioral patterns geared to parental care and feeding (e.g., food begging behaviors) and a lack of thermoregulatory responses to cooling for much of the nestling period. Although most birds fall into one of these general developmental categories, there is a continuum along which some birds are intermediate and are categorized as semi-altricial or semi-precocial (see Starck and Ricklefs 1998 for altricial and precocial patterns; see McNabb and Olson 1996 for patterns of thermoregulatory development in precocial and altricial birds).

539. Thyroid hormones regulate that part of metabolism that is associated with the heat increment that distinguishes homeothermic birds and mammals from poikilothermic vertebrates (Danforth and Burger 1984; Danforth 1986). Associated with this, the developmental patterns of thyroid function are markedly different in precocial vs. altricial, birds and mammals, the two homeothermic classes of vertebrates (review, McNabb 1989). In precocial birds, such as galliform chickens and quail, the thyroid gland differentiates during the first few days of incubation and comes under pituitary control at about 1/3 to 1/2 of incubation (reviews, Thommes 1987; McNabb 1987). During the latter half of incubation, circulating T_4 rises steadily concurrent with increases in thyroid gland function which outpace body growth by several fold (based on radioiodine uptake studies and the accumulation of thyroidal hormone stores; McNabb et al. 1981; McNichols and McNabb 1988). Just prior to hatching, HPT axis signals (Gregory et al. 1998) trigger marked increases in T_4 release resulting in a dramatic peak in circulating T_4 concentrations during the perihatch period (review, Thommes 1987). Circulating T_3 concentrations also peak during this time but are slightly behind those of T_4 (McNabb et al. 1981; McNabb and Hughes 1983). The spike in T_3 results from marked increases in the activity of Type I hepatic 5'-deiodinase (5'D I; Decuypere et al. 1982; Freeman and McNabb 1991) which is the main supplier of T_3 to the circulation in mammals (Leonard and Visser 1986) and appears to perform the same function in birds. Concurrent with this perihatch increase in 5'D I, there is a decrease in hepatic 5-deiodinase (5D), a deactivating pathway that converts T_4 to inactive reverse-T_3 (Galton and Hiebert 1987; Darras et al. 1992). It is often stated that this deactivating pathway is protective of T_3 toxicity to embryos. However, whether or not this is the key function of 5D activity in embryos is difficult to verify and convincing experimental work is lacking. Circumstantial evidence suggests that the peaks in circulating T_4 and T_3 concentrations are associated with the initiation of thermoregulatory responses in precocial birds in response to cooling during the hatching process (Freeman 1970, 1971). Altricial species which are poikilothermic at this time do not have a perihatch peak in thyroid hormones (see below). After the perihatch period, at a few days of age, circulating T_4 and T_4 both decrease markedly in precocial chicks, then increase moderately to reach adult levels. The plasma T_4 and T_3 concentrations in juvenile and adult birds are characteristically lower than those of the perihatch peak. The pattern described above is consistent for precocial birds that have been investigated (chickens, Thommes and Hylka 1977; Japanese quail, McNabb et al. 1981; turkeys, Christensen et al. 1982). To date, studies of

the patterns of thyroid development in precocial birds have been limited to studies of galliforms, and precocial species from other avian orders have not been investigated.

540. In contrast to precocial species, altricial birds show very little histological or functional thyroid development during embryonic life or the perihatch period. In altricial birds neither the increase in circulating T_4 during late incubation nor the perihatch peak in thyroid hormones that occurs in precocial birds, are present. In altricial ring doves, circulating thyroid hormone concentrations are very low and the thyroid does not respond to TSH until several days posthatch (McNichols and McNabb 1988). The functional capacity of the thyroid, and its hormone storage and release of hormones to the circulation then increases gradually during the first 3 weeks of posthatch life. Circulating thyroid hormone concentrations plateau after 2-3 weeks concurrent with the transition from poikilothermy to homeothermy (McNichols and McNabb 1988). As indicated above, there is no perihatch peak in circulating thyroid hormones in altricial birds which are essentially poikilothermic during this time. Several altricial species from different avian orders, have been investigated and show consistent patterns of circulating thyroid hormones during development (ring doves, McNabb and Cheng 1985; starlings, Schew et al. 1996; Vyboh et al. 1996; red-winged blackbirds, Olson et al. 1999; review, McNabb and Olson 1996). For a broader review of all growth-associated hormone patterns in precocial and altricial birds, see McNabb et al. (1998). It should also be noted that the general patterns of thyroid development in precocial and altricial birds are like those in precocial and altricial mammals (review, McNabb 1989).

7.1.4.2 Diurnal Patterns

541. Studies of diurnal patterns indicate that circulating T_4 concentrations rise and peak during the dark phase of the diurnal cycle and fall during the light phase in chickens, quail and ducks. The plasma T_3 pattern is the opposite to that of T_4 (review, Wentworth and Ringer 1986; Cogburn and Freeman 1987). These patterns for T_4 and T_3 are consistent with the idea that the release of thyroid hormones (almost entirely T_4) from the thyroid gland is highest during the dark period and extrathyroidal T_4 to T_3 conversion is highest during the light period. However, neither thyroidal T_4 release nor 5'D I activity have been measured over the 24 hour daily period. Food intake increases 5'D I activity and in fasting chickens the rhythmic pattern of T_3 disappears, so daytime food intake is a key factor involved in higher circulating T_3 concentrations in the light than in the dark phase (review Decuypere and Kühn 1988). Seasonal patterns of circulating hormones will be described in section 7.3.

7.1.4.3 Transport Proteins

542. In birds, the main thyroid hormone binding proteins in blood are transthyretin (TTR or thyroxine-binding prealbumin) and albumin. Birds lack a specific T_4-binding protein such as the thyroxine-binding-globulin (TBG) found in large mammals (review, Wentworth and Ringer 1986). In Japanese quail and chicken plasma, *in vitro* studies with labeled T_4 show the relative proportions of T_4 bound by these proteins are: 17-32% to TTR, 66-75% bound to albumin, and the remaining few percent bound to other plasma proteins (Davison et al. 1978; Tanabe et al. 1969; McNabb and Hughes 1983). In these same studies T_3 bound primarily to albumin and globulins. Free hormone RIAs suggest the free T_4 and T_3 concentrations in avian plasma are similar to those in mammalian plasma (McNabb and Hughes 1983). During development the binding proteins in avian plasma appear to modulate hormone availability at different developmental stages (McNabb and Hughes 1983; McNabb et al. 1984; Nishiguchi and Hoshino 1993).

543. Recently Schreiber's laboratory has developed methods that overcome some of the problems with variability that have been characteristic of studies of TTR binding affinity and has used those methods in extensive studies of TTR binding across the vertebrate classes. In addition to questioning some of the past generalities about mammalian TTR, these studies indicate that avian TTR (chicken, pigeon, ostrich, emu) has approximately twice the affinity for T_3 that it has for T_4. This results from avian TTR having about 4X

higher affinity for T_3 than is the case for mammalian TTR (Chang et al. 1999). This finding of higher T_3 affinity is in marked contrast to the situation in mammals in which TTR has approximately 4X the affinity for T_4 than it has for T_3 (review, Schreiber 2002).

544. In mammals, binding proteins are generally considered to serve as an extrathyroidal hormone store, to help regulate hormone supply to the tissues, and to help regulate hormone distribution within organs (Schreiber 2002). Some studies are suggestive of binding proteins modulating tissue hormone supplies in birds, e.g. in development in quail and doves (McNabb and Hughes 1983; McNabb et al. 1984; Spiers and Ringer 1984) and in diurnal patterns in ducks (Harvey et al. 1980). TTR is produced in the choriod plexus (as well as the liver) of both birds and mammals and is involved in regulating the transport of thyroid hormones into the central nervous system (Southwell et al. 1991; review Schreiber 2002). New physiologically oriented studies of the role(s) of binding proteins in birds are needed, especially to further clarify its role in binding of T_3.

7.1.4.4 Factors Affecting Thyroid Hormone Concentrations

545. Adult birds usually have plasma or serum T_4 concentrations in the range of 5-15 ng/ml (6-19 pmol/ml) and T_3 concentrations in the range of 0.5-4 ng/ml (0.7-1.5 pmol/ml; reviews, Astier 1980; McNabb 2000). Compared to mammals, birds have similar plasma T_3 concentrations but much lower plasma T_4 concentrations. In addition to the developmental patterns of thyroid hormones described in 7.1.4.1 and the diurnal patterns described in 7.1.4.2, circulating thyroid hormones also vary with food availability, the nature of the food sources utilized, the adequacy of iodine availability, the temperature conditions and, on longer time scales, with season and reproductive condition. Food availability and temperature appear to have the largest effects on plasma thyroid hormones. Both short term fasting and longer term starvation are associated with decreases in thyroid hormones, especially in T_3, in both birds and mammals (review, Eales 1988).

546. In birds, as in vertebrates in general, complete fasting (starvation) decreases circulating T_3 concentrations but may have differential effects on T_4 (plasma T_4 decreases in mammals but usually increases or remains constant in adult chickens; reviews Eales 1988; Darras et al. 1995). In most vertebrates the fasting induced decreases in T_3 appear to be due to decreases in hepatic T_4 to T_3 deiodination (review Eales 1988). However, in partial food restriction, an increase in the deiodination enzymes that degrade T_3 may play a key role in plasma T_3 decreases in both chickens and rats (Darras et al. 1995; see 7.1.4.6 for more detail about the roles of deiodinases in regulating circulating thyroid hormones). Refeeding restores T_3 concentrations at rates related to the caloric content of the food. Other hormones such as insulin and glucagon, which are involved in the regulation of glucose can also play additional roles (e.g. glucagon stimulates T_4 to T_3 conversion; Eales 1988).

547. Temperature patterns can modulate the hormone concentrations within the diurnal cycles. In chickens, cold temperatures increase and warm temperatures decrease plasma T_3 and the effects on T_4 are generally in the opposite direction (Cogburn and Freeman 1987). The changes in circulating T_3 in cold exposure reflect cold-induced increases in T_4 to T_3 deiodination in peripheral tissues (Rudas and Pethes 1984, 1986).

7.1.4.5 Thyroid Hormone Transport Kinetics

548. Compared to mammals, relatively little is known of thyroid hormone kinetics in birds. A number of studies from the 1950s and 1960s found apparent half-lives in several avian species (chickens, ducks, Japanese and Bobwhite quail) that were similar for T_4 and T_3 and that were short (3-9 hours) compared to those in mammals using the same techniques. Many aspects of the methods used for these studies are now considered inadequate for accurate half-life measurements, so the specific values should be considered

cautiously until better methods are used (review McNabb 2000). There is some evidence that cold temperatures decrease the half-life of T_4 in galliform birds (review McNabb 2000) and this would be consistent with the increase in T_4 to T_3 conversion that occurs at cold temperatures (Rudas and Pethes 1984, 1986).

549. Specific transport of thyroid hormones into the central nervous system is thought to be mediated by TTR which is produced in the choroid plexus in birds, as in mammals (Southwell et al. 1991; Schreiber 2002). Studies of adaptive changes in the uptake of thyroid hormones with altered thyroid states offer physiological support for this idea (Rudas 1989; Rudas and Bartha 1993; Rudas et al. 1994).

7.1.4.6 Deiodination Enzymes

550. The patterns and proportions of T_4 and T_3 in the circulation depend not only on hormone production and secretion by the thyroid gland, but also on deiodinase enzymes present in extrathyroidal tissues. The extreme predominance of T_4 over T_3 in the avian thyroid gland (see 7.1.2.2 and 7.1.2.3) and the much higher T_3/T_4 ratios in the circulation, suggest that in birds as in mammals, most T_3 is produced extrathyroidally. Three key deiodination pathways need to be considered: (1) Type I deiodinase (referred to as 5'D I) which converts T_4 to T_3 (outer ring deiodination) as well as T_4 to rT_3 (inner ring deiodination), (2) Type II deiodinase (5'D II) which converts T_4 to T_3 (outer ring deiodination) and (3) Type III deiodinase (5D III) which deactivates T_3 to diiodothyronine (inner ring deiodination). Thus, Type I and Type II 5' D both activate T_4 to T_3 whereas Type III deactivates T_3 to inactive T_2. In addition Type I can "dispose of " T_4 by converting it to rT_3 (i.e., "prevent" T_3 production). In general, many of the key patterns and characteristics of deiodinases in birds appear to be similar to those described for mammals (reviews, McNabb 1992; Kühn et al. 1995). Type I activity is present in liver, kidney, and small intestine of chickens, Japanese quail and ring doves, Type II is present in brain of chickens and herring gulls and Type III is present in liver of chickens (Borges et al. 1980; Galton and Hiebert 1987; Freeman and McNabb 1991; Darras et al. 1992; Rudas et al. 1993; Suvarna et al. 1993; Fowler 2001).

551. Studies of the characteristics of the three types of avian deiodinases have shown them to be very similar to the deiodinases in mammals (Type I, Freeman and McNabb 1991; Darras et al. 1992; Type II, Rudas et al. 1993; Type III, Darras et al. 1992). Because of these similarities, it generally is assumed that in adult birds hepatic 5'D I is playing the same major role in supplying most of the T_3 for the circulation in birds as in mammals. Recent studies of liver T_4 and T_3 content in fed vs. fasted birds provide evidence that the liver is an important supplier of T_3 to the circulation (Reyns et al. 2002). However, the relative importance of 5'D I in kidney, which also is important in generating T_3 for supply to the plasma in mammals, has not been investigated.

552. Some important aspects of the changes in deiodinases with different developmental and physiological states also are similar in birds and mammals, for example, in the liver of chicken embryos most T_4 is deiodinated to rT_3 and little T_4 is converted to T_3 (Borges et al. 1980; Galton and Hiebert 1987). Because there also is an active Type III 5D present at this time, any T_3 that is produced is converted to T_2 (Darras et al. 1992). Together this combination of deiodinase activities results in very low circulating T_3 concentrations until the perihatch period when there are marked increases in 5'D I during the perihatch period (quail, Freeman and McNabb 1991; chickens, Darras et al. 1992) and concurrent decreases in 5D III that are largely mediated by growth hormone (chickens Darras et al. 1992). The expression and activity of the different deiodinases in many tissues has been investigated during the last week of embryonic chick development (Van der Geyten et al. 2002). Consistent with the general picture that has developed from enzyme activity studies (see above) this survey found 5D III to be the most widely expressed; it occurred in all tissues examined, thyroid, lung, brain, pituitary, heart, liver, spleen, gonads, skin, muscle, intestine, Bursa of Fabricius and kidney. The expression and activity of 5'D I also were widespread but 5'D I was not found in brain, thyroid, muscle or skin. 5'D II expression and activity were only present in brain at this

stage of development (Van der Geyten et al. 2002). Other examples of how changes in deiodinase activities alter circulating thyroid hormone concentrations with different physiological states are seen during fasting, partial food restriction and exposure to cold (see 7.1.4.4).

553. Brain 5'D II also appears to be very similar in its physiological roles in birds (Rudas et al. 1993) and mammals (Kaplan 1986; Leonard and Visser 1986). The activity of 5'D II shows adaptive patterns that protect the central nervous system from extremes of high and low thyroid hormone exposure. Thus, in response to low circulating thyroid hormones in hypothyroidism, 5'D II increases to maintain euthyroid T_3 levels in the central nervous system. Conversely, in response to high circulating thyroid hormones in hyperthyroidism, 5'D II decreases to maintain euthyroid T_3 levels in the central nervous system (Kaplan 1986). The studies of Rudas et al. (1993, 1994) and Rudas and Bartha (1993) in young chickens indicate that 5'D II and the movements of thyroid hormones in and out of the central nervous system are regulated in response to changes in thyroid status (Rudas 1989; Gereben et al. 1998). The 5'D II gene of chickens has been cloned and the increase in its expression in specific brain regions during hypothyroidism has been demonstrated. In addition, in contrast to mammals, in chickens 5'D II mRNA and enzyme activity are present in liver and may play a role in avian circulating T_3 production (Gereben et al. 1999).

554. Tissue-specific deiodination patterns also may play a role in the effects of thyroid hormones on tissue development or physiological or biochemical activities. For example, differences in 5'D I activity in the intestine of high and low weight lines of chickens suggest local T_3 supply may be important in regulating the timing of tissue differentiation (Suvarna et al. 1993). The recent study of Van der Geyten et al. (2002; see description above) illustrates the range of deiodination patterns in different tissues in avian embryos. These different combinations of deiodinases in different tissues have the potential for tissue-specific regulation of thyroid hormone effects.

7.1.4.7 Thyroid Hormone Action

555. There is considerable evidence that T_3 is the metabolically active hormone accounting for most thyroid hormone action in mammals. The main reason for considering T_3 the metabolically active hormone in mammals is the much higher affinity of the thyroid receptor (TR) for T_3 than T_4 (see Section 3.8.1) and because studies have shown that most occupied TR sites *in vivo* are occupied by T_3. T_4 appears to be primarily a prohormone (which is deiodinated to T_3), although T_4 can bind to thyroid receptors and initiate tissue responses. Overall, the physiological potency of T_3 is much higher than that of T_4 in mammals (Engler and Burger 1984). In contrast to this well documented picture of T_3 as the main metabolic hormone in mammals, there is a body of evidence published between 1950 and 1970 that suggests T_4 and T_3 are of essentially equal physiological potency in birds (review, McNabb 2000). These studies assessed a wide variety of physiological, thyroid hormone-responsive endpoints (such as organismal and tissue oxygen consumption, heart rate, feather growth, and amino acid uptake in growing tissues) and found similar or equal potency of T_3 and T_4 in birds (review, McNabb 2000), a result which is in dramatic contrast to the results of similar studies in mammals. Essentially all of these studies were done prior to the discovery that thyroid receptors have their highest affinity for T_3 and prior to when most knowledge of deiodinases developed. Neither the studies on birds nor those on mammals attempted to block deiodinase activities. However, it is hard to see how this could account for the differences in potency between birds and mammals in similar studies. At present, it is not understood why T_4 and T_3 appear to be essentially equipotent in birds.

556. Birds have TRs that are essentially identical to those of mammals with respect to their biochemical characteristics, including having higher affinity for T_3 than T_4 (Weirich and McNabb 1984; Bellabarba et al. 1988). In addition the same receptor isoforms are found in both birds and mammals (see below). Because of these receptor similarities, many congruencies in deiodinase activity in birds and mammals (see 7.1.4.6), and the higher circulating T_3/T_4 ratio in birds than in mammals, it is generally

assumed that T_3 is responsible for most actions of thyroid hormones in birds. However, there are no data available on the proportion of receptors occupied by T_3 *in vivo* so some key information that would provide direct support for this assumption is lacking.

557. As in mammals (Chapter 3, Sections 3.7.1-3.7.3), the expression of TRα and TRβ isoforms differs by tissue in embryonic and early posthatch chickens and there are different developmental patterns for the different TR isoforms (Forrest et al. 1990). Specifically, TRα is widely expressed in different parts of the chick brain throughout embryonic development and for the first three weeks posthatch (Forrest et al. 1990) whereas TRβ expression increases sharply at day 19 of the 21-day incubation period, coincident with the thyroid hormone-sensitive period of brain development (Forrest et al. 2001). However, this study also suggested early embryonic effects of both forms of the receptor at other times in brain development when the effects of thyroid hormone are not well understood. TRα also was found in all of the 14 tissues examined during embryonic and posthatch development, although the presence of TRβ was restricted to brain, eye, lung, yolk sac and kidney and the patterns of its expression differed with developmental age (Forrest et al. 1990). Overall, these data suggest general early developmental roles for TRα and specific developmental roles for TRβ, as is the case for this isoform in other vertebrate classes (see Chapters 3 and 6 of this review). Additional evidence that TRα is important in very early embryonic development comes from the studies of Flamant and Samarut (1998) who found a low level of TRα expression initially followed by an increase in expression in neuroectoderm during neurulation in the chicken embryos prior to incubation. They demonstrated that maternal T_3 was released from egg yolk prior to gastrulation and that the pattern of T_3 tissue distribution and the effects of T_3 alterations were suggestive of TRα , like those of RXR, playing a role in embryonic development prior to the onset of embryonic thyroid gland function.

558. Several studies have investigated receptor isoform distribution and changes in different tissues in relation to thyroid status. Brain receptor numbers change, in an adaptive manner, in response to altered thyroid states after hatch in precocial birds as they are known to do in young mammals. Both TRα and TRβ are increased in the brain in young chickens in response to developing hypothyroidism following thyroidectomy. This receptor response is part of a suite of adaptive actions that include increased T_3 uptake (Gereben et al. 1998) and increases in brain 5'D II activity (see Section 7.1.4.6 above) in response to hypothyroidism.

559. Receptor isoforms also have been investigated in cardiac, pectoralis and semimembranous leg muscle, as well as in liver, pituitary and cerebral cortex in ducklings rendered hypo- or hyperthyroid by methimazole or methimazole + T_4 or T_3. TRα expression was present in all the tissues sampled but was unaffected by any of the treatments. In contrast, in association with lower leg muscle growth in the methimazole treated birds, the expression of TRβ 1 was depressed significantly compared to controls; TRα expression in pectoralis and cardiac muscle was not significantly altered by this treatment although the growth of these muscles was depressed. Thyroid hormone treatment elevated TRβ 1 expression in all three muscles. TRβ 2 was only expressed in the pituitary in this study (Bishop et al. 2000). In the context of HPT axis activity, the TRβ 2 is active in the feedback inhibition of T_3 on hypothalamic TRH gene expression in chicken embryos (Lezoualc'h et al. 1992, 1994).

7.1.4.8 Thyroid Hormone Metabolism

560. Thyroid hormone metabolism (i.e., degradation) by pathways other than deiodination can involve conjugation, deamination, decarboxylation or cleavage at the ether linkage between the aromatic rings. In mammals, for T_4 degradation, deiodination has been estimated to account for about 80% and these other degradative pathways account for <20% (Engler and Burger 1984). Both T_4 and T_3 readily form conjugates of sulfates and glucuronides. The major conjugation of T_4 is the esterification of the phenolic hydroxyl with glucuronide. For T_3, the major conjugation is with sulfate (Sekura et al. 1981). Although a

large proportion of the T_4 produced by the thyroid appears as conjugates (of glucuronide or sulfate) in bile, there is evidence in some experimental mammals that as much as 40-60% of this sulfoconjugate is deconjugated by intestinal bacteria and reabsorbed (Kung et al. 1988). There is evidence in rats that sulfoconjugation followed by deiodination accounts for the largest proportion of T_3 degradation (Mol and Visser 1985a,b; deHerder et al. 1988). Surprisingly, studies of thyroid hormone distribution in rats found no thyroid hormone conjugates in the feces (DiStefano 1988). This argues for all conjugated thyroid hormones being recovered in the intestine at least in "normal" animals. However, in PCB treated rats there is good evidence that increases in UDP-GT are responsible for marked decreases in circulating T_4 (Barter and Klaassen 1992a, 1994) suggesting that UDP-GT induction does increase hormone excretion as well as metabolism.

561. Uridine diphosphate glucuronosyl transferase (UDP-GT) is present in avian liver based on *in vitro* activity toward *p*-nitrophenol, a substrate thought to reflect the activity of most or all of the isoforms of UDP-GT (Japanese quail, Riviere et al. 1978; chicken embryos, McCleary 2001; adult chickens and ostriches, Amsallem-Holtzman and Ben-Zvi 1997). The activity of UDP-GT in birds varies considerably in these species and is much lower than that of rats (comparison of Amsallem-Holtzman and Ben-Zvi 1997). This enzyme is of particular concern in studies of thyroid disruption by chemical contaminants because studies with laboratory rats show that chemicals such as PCBs induce UDP-GT, thereby enhancing the glucuronidation of T_4 and its excretion in bile (Barter and Klaassen 1992b). One study has shown increased UDP-GT activity in Japanese quail liver at a dose of 5 mg Phenoclor/bird/day (Riviere et al. 1978).

7.1.4.9 Dynamic Relationships among Levels Within the HPT Axis

562. When decreases in circulating thyroid hormones feedback to the HPT axis a series of adjustments "attempt to" compensate and restore circulating hormones to euthyroid levels. However, the unique hormone storage capacity of the thyroid gland makes this compensation somewhat different from that in other endocrine axes where the final endocrine organ does not possess appreciable storage capacity. In birds, as in other vertebrates (Section 7.1), if circulating thyroid hormones decrease, negative feedback will result in increased TSH release from the pituitary with consequent stimulation of thyroid gland growth and function (thyroidal iodide uptake and the capacity to produce, store and release hormones). Most important to early responses is that the stored hormones in thyroid glands are available for release to restore circulating thyroid hormone concentrations. However, this restoration may be transient; depending on the type and magnitude of the problem that caused the original depletion, circulating hormones may again decrease, resulting in TSH release and more release of stored thyroid hormones. Thus, in those cases where increased thyroid gland function cannot compensate, a cyclic pattern of decreases in circulating hormones and release of stored hormones can occur with resulting depletion of thyroid gland hormone stores. This type of cyclic pattern of responses occurs in response to iodine deficiency in humans (Delange and Ermans 1996) and appears to be the case in both quail (McNabb et al. 2004) and rats (York et al. 2001) exposed to perchlorate which interferes with thyroidal iodide uptake. Such cyclic patterns of HPT axis response appear to be a key reason why measurements of plasma thyroid hormones often are highly variable in studies of endocrine disruption by chemicals (Section 7.6.2.1).

7.1.5 Thyroid Hormone Negative Feedback on the HPT Axis

7.1.5.1 Thyroid Hormone Negative Feedback on the Pituitary

563. Studies that decrease or enhance circulating thyroid hormones have demonstrated negative feedback effects on the avian pituitary (review, Scanes 2000). In mammals, most of these feedback effects are from T_4 which enters the pituitary and is then deiodinated by 5'D II to produce T_3, which in turn binds to TRβ and inhibits TSH production and release (Silva and Larsen 1977). TRβ 2 expression is present in

the pituitary in ducklings (Bishop et al. 2000). The gene for 5'D II is present in embryonic chicken cDNA libraries (Sun et al. 1998) but surprisingly 5'D II mRNA transcripts were not detectable in late incubation in the study of Van der Geyten (2002). These studies indicate that avian embryonic deiodination of T_4 to T_3 in brain and perhaps pituitary are similar to those in mammals.

7.1.5.2 Thyroid Hormone Negative Feedback on the Hypothalamus

564. Molecular studies have demonstrated that increased T_3 results in TRβ mediated inhibition of TRH gene expression in hypothalamic neurons from chick embryos but that TR α does not play a role in this negative feedback (Lezoualc'h et al. 1992). Immunocytochemical studies of hypothalamic neurons from day 6 chicken embryos, maintained in culture, possess TRs suggesting that the negative feedback effect of T_3 on TRs matures relatively early and prior to the time when there is linking of the HPT axis function (Lezoualc'h et al. 1994).

7.1.6 Summary of Differences between Avian and Mammalian HPT Axis

565. In general, there are few differences between birds and mammals in thyroid function and its control. Some differences are: differences in the iodination of thyroglobulin in the representatives of those classes that have been tested to date, differences in the thyroid hormone binding proteins between birds and large mammals, and shorter half-lives of thyroid hormone in birds than in mammals.

7.2 Roles of Thyroid Hormones in Avian Development

7.2.1 Effects on Growth

7.2.1.1 Interactions between the Thyroid and Growth Axes

566. Thyroid hormones are required for growth in birds, and within some range, growth is related to thyroid hormone exposure. However, at circulating thyroid hormone concentrations both above and below this range, growth is decreased (reviews, King and May 1984; McNabb and King 1993; Cogburn et al. 2000). Most of the work in this area has been in chickens because of the economic importance of optimizing growth in poultry. Thyroid hormones appear to act on growth in a permissive or indirect way in conjunction with other hormones of the growth axis. In birds, as in mammals, most of the end organ growth is thought to result from the effects of hepatic insulin-like growth factors (IGFs) whose secretion is largely under the control of growth hormone (GH) from the pituitary (Cogburn et al. 2000). Chickens with the sex-linked dwarf gene have provided a useful model for examining the effects of thyroid hormones in avian growth. These birds have low plasma T_3, normal or high plasma T_4, no alterations in binding proteins and only slight decreases in thyroid gland function. The defect responsible for the decrease in circulating T_3 is a deficiency in hepatic 5'D I (review, Decuypere and Kühn 1988).

567. There are interactions in which the HPT axis affects the growth axis, with TRH stimulating, and somatostatin inhibiting, GH release (review, Scanes 2000). In contrast to mammals (in which thyroid hormone stimulates GH secretion), in birds, thyroid hormones decrease GH secretion by effects on pituitary somatotropes and by negative feedback effects on TRH (Scanes 2000). Recently thyroid hormones have been shown to affect the differentiation and abundance of somatotrophs in chicken embryos *in vivo* (Liu et al. 2004) and *in vitro* studies indicate this stimulation requires synergistic interactions with corticosterone (Liu and Porter 2003).

568. There also are interactions between the thyroid and GH axes in the opposite direction; GH has regulatory effects on some aspects of thyroid function. Increases in GH just prior to hatching stimulate the increase in plasma T_3 during the perihatch period in precocial chickens. The GH increase causes marked

and rapid decreases in 5D III activity (decreased T_3 degradation) and cause a slower increase in 5'D I activity (increased T_3 production from T_4). Together these alterations in deiodination result in the perihatch T_3 peak (see Section 7.1.4.6). The effects of GH on 5D III also are present in immature growing broiler chickens (Vasilatos-Younken et al. 2000). Glucocorticoids, which also may influence deiodinases, increase late in incubation/gestation, but in contrast to mammals, they appear not to be an important factor in the changes in deiodination activity at this time in precocial birds (see review in Darras et al. 1995). The effects of GH on deiodinases vary with deiodinase type and tissue; GH decreases hepatic 5D III (effect at the level of transcription) but does not alter 5'D II in the brain (Van der Geyten et al. 2001). GH receptors are present in the thyroid and *in vitro* studies indicate that GH decreases T_4 release from the thyroid and may directly stimulate thyroid gland growth (Hull et al. 1995).

569. Nutritional status also may play a role in thyroid and growth axis interactions. Long term food restriction results in decreases in plasma T_3 but increases in plasma T_4, GH and IGFs (Bruggeman et al. 1997). The changes in plasma GH as well as in hepatic GH receptor numbers may be important in altering deiodination patterns and subsequent effects on plasma T_3. This sequence of events has been shown to be important in the restoration of plasma T_3 following refeeding (Buyse et al. 2002).

7.2.1.2 Embryonic, Perihatch and Posthatch Growth

570. The effects of thyroid hormones on body growth of precocial birds during the latter half of embryonic life as well as posthatch have been demonstrated using thyroid inhibitors, iodine deficiency and T_4 supplementation (reviews, King and May 1984; McNabb and King 1993; McNabb et al. 1998). It is less clear whether thyroid hormones are required for growth during the early parts of embryonic life. Hens deposit thyroid hormones in their eggs in relation to their own thyroid status so maternal hormones are available in the egg prior to the time when the embryonic thyroid is producing and releasing appreciable thyroid hormone (review, McNabb and Wilson 1997). Maternal hormones in the egg can affect embryonic tissue growth; increased maternal hormone content of eggs from T_4 supplemented hens was associated with increased pelvic cartilage growth and differentiation by late incubation in Japanese quail (Wilson and McNabb 1997). Pelvic cartilage is a tissue where both T_3 and insulin-like growth factors are required for differentiation and growth (Burch and Lebovitz 1982). There is an extensive literature on many factors that affect growth in poultry and a number of studies have attempted to manipulate the hormones of the HPT axis, largely without success, in attempts to find hormonal strategies for augmenting poultry growth.

571. Little is known of the relationships between thyroid hormones and growth in altricial birds. In a number of altricial species circulating thyroid hormone concentrations are very low during embryonic and early posthatch life then gradually increase until they reach levels like those in adults by the time of fledgling (see Section 7.1.4.1). Many altricial birds grow very rapidly during the early posthatch period when thyroid hormones are very low and these essentially poikilothermic chicks are investing energy in growth but not in thermoregulation (McNabb and Olson 1996). In altricial starlings, growth during this early posthatch period is associated with transient increases, then decreases, in GH and IGFs and gradual, sustained increases in T_4 and T_3 in the circulation (Schew et al. 1996). Studies of plasma GH and IGF concentrations in several altricial species indicate that both these hormones are high in the early posthatch period (up to about 10 days) then fall to low levels in both altricial and precocial birds (McNabb et al. 1998).

7.2.2 Induction of Tissue-Specific Differentiation/Maturation

572. Thyroid hormones are involved in the differentiation and maturation of many body systems in all classes of vertebrates. The systems that are most dependent on THs during development are the central nervous system, the skeletal system, the heart and body musculature. Many if not all of these developmental events are initiated by T_3 binding to TRs resulting in transcription of specific genes and the

transduction of specific structural or enzymatic proteins (see Section 3.8). In general in birds, thyroid hormones appear to directly trigger some of the same specific differentiation and maturation events as in mammals, although far fewer examples have been addressed.

573. The older literature on thyroid hormone effects that point to specific effects of thyroid hormones on differentiation in skeletal components and muscle have been reviewed by King and May (1984) and King et al. (1987). The contractile characteristics and metabolic activity of three fast and slow muscle types are closely correlated with T_3 receptor numbers during development (Dainat et al. 1986). These studies of receptor development suggest that the effects of thyroid hormones on developmental events are modulated by several factors including T_3 availability, receptor numbers, receptor binding affinities, and the linking of receptors to physiological effects (see below).

574. *In vitro* studies of embryonic chick cartilage have been used to examine some of the biochemical events involved in the hormonal control of skeletal development (Burch and Lebovitz 1982). Specifically IGFs appear to trigger T_3 stimulation of cartilage growth by chondrocyte proliferation but the thyroid hormone stimulation of chondrocyte differentiation is independent of IGFs (Burch and Van Wyk 1987). Other specific differentiation/maturation events triggered by thyroid hormones in perihatch chickens are the maturation of lung tissue just prior to the initiation of pulmonary respiration (Wittmann et al. 1983) and the maturation of intestinal function just prior to feeding (Black and Moog 1978; Black 1988).

7.2.2.1 Brain Development and Architecture

575. Thyroid hormones are critical to the establishment of brain architecture during central nervous system development in vertebrates. Thyroid hormones are required for the development of cellular branching processes that lead to the elaborate synaptic interconnections between brain neurons and the architecture of each brain region (for a comparative review see McNabb 1992). Thus altered thyroid states during development are likely to result in serious, permanent effects on central nervous system function. Essentially all of the recent cellular and molecular work in this area has been done on rats and it is reviewed in detail in Chapter 4, sections 4.3 and 4.4. Only the very limited studies on birds will be covered in this section.

576. Studies focused on TR provide evidence that maternal T_3 from yolk and TRα (but not TRβ) play a role in the early stages of nervous system differentiation in chicken embryos prior to the onset of thyroid gland function (Flamant and Samarut 1998). In cultures of quail neural crest cells, retinoic acid and T_3 interact (the first is stimulatory and the second inhibitory) in the control of adrenergic cell development (Rockwood and Maxwell 1996). Surveys of the presence of TRβ in embryonic chicken brain are suggestive of roles of this receptor isoform in later aspects of avian brain development especially in the last few days before hatch in this precocial species (section 7.1.4.7).

577. Some investigations of T_3 effects on mitosis, gene expression and apoptosis have used chicken embryos. For example, between days 6 and 11 of the 21-day incubation period, exogenous T_3 stimulates mitosis followed by changes in the degree and timing of apoptosis in the optic lobes of chicken embryos. However, in this case the effects of elevated T_3 were transient (Ghorbel et al. 1997).

578. Morphometric studies of brain development in birds, exposed to several persistent types of persistent polyhalogenated aromatic hydrocarbons that alter thyroid function, have shown grossly asymmetric brain development (Henshel et al. 1997a,b; Henshel 1998). This technique may have promise in investigations of developmental effects of thyroid disruption (section 7.6.3.5) but to date these studies have not measured thyroid variables to attempt to correlate them with altered brain development.

7.2.2.2 Neuronal Turnover in Adult Birds

579. A great deal of attention has focused on the neurogenesis that occurs throughout adult life in the telencephalon of songbirds. Most of the attention has been on the role of gonadal steroids in this aspect of brain function (Rasika et al. 1994; Hidalgo et al. 1995; Smith et al. 1997; Bernard and Ball 1997). However, thyroid hormones are among the hormones that appear to play either independent or interactive roles in the plasticity exhibited by this part of the songbird brain. Circulating thyroid hormones are high during molt in association with the highest rates of cell turnover in the high vocal center (HVC; Kirn et al. 1994). Recently Tekumalla et al. (2002) investigated the effects of T_4 treatment in adult zebra finches and found increased neuronal turnover and a decreased number of HVC neurons. The decrease in cell numbers was due to altered cell survival, specifically increased cell death in regions where they detected the presence of TRs. The effects of thyroid hormone treatment were transient and subsequent cell proliferation was not compensatory. Some species of songbirds show seasonal changes in HVC neuron numbers (Tramontin and Brenowitz 2000) and seasonal changes in thyroid hormones. Overall, these findings argue for thyroid hormones playing a role in regulating the timing of song learning and production (Tekumalla et al. 2002).

7.2.2.3 Skeletal System (cartilage and bone)

580. Thyroid hormones affect the development of the skeletal system through effects on the initiation and fusion of ossification centers as well as on bone elongation. The hormonal control of postnatal skeletal growth in mammals involves both growth-related and thyroid hormones. GH appears to be primarily responsible, through IGF actions on cell proliferation, for cartilage growth in the epiphyseal plate of long bones. Thyroid hormones stimulate the maturation of cartilage cells, and the deposition of the matrix and its mineralization. Prenatal skeletal development is thyroid hormone dependent in mammals, but some early aspects of skeletal development in altricial rats do not appear to require either thyroid hormones or GH (reviews, Legrand 1986; Schwartz 1983). Embryonic chick pelvic cartilage has been used as a model system for investigating some of the effects of thyroid hormones and the interactions between thyroid and growth-related hormones in the control of skeletal development. Thyroid hormone stimulation of cartilage growth is initiated by IGFs although T_3 stimulation of maturation (differentiation into hypertrophic chondrocytes) is independent of IGFs (Burch and Lebovitz 1982; Burch and Van Wyk 1987). Pelvic cartilage growth and alkaline phosphatase activity (which indicates differentiation) effectively reflected increased maternal T_4 exposure of embryos *in ovo* in eggs from hens treated with T_4 (Wilson and McNabb 1997).

7.2.2.4 Muscle

581. Skeletal muscle comprises a large proportion of body mass so it represents a large proportion of body growth. In mammals, thyroid hormones are necessary for normal skeletal and cardiac muscle growth and have a number of specific, direct effects on the maturation of muscle cells. Thyroidectomy at birth in rats, which are altricial and have most thyroid development after birth, results in marked deficiencies in skeletal muscle mass and alterations in muscle proteins, with the most extreme effects on myosin. There is evidence in both rats and chickens that thyroid hormone replacement reverses the effects of hypothyroidism on myosin, that GH administration reverses the effects on muscle weight but not those on myosin, and that thyroid hormones plus GH administration results in a synergistic effect with larger muscle weight than with either hormone alone (reviews, Legrand 1986; Scanes et al. 1986). Thyroid hormones alone do not result in normal growth if GH is not present, although they do stimulate increases in protein synthesis. Thus, thyroid hormones do not seem to stimulate the proliferative aspects of muscle growth, because DNA synthesis and satellite cell formation are not increased. These latter effects appear to be the most important actions of GH in skeletal muscle (Legrand 1986).

582. In birds, as in mammals, thyroid hormones are required for the differentiation of muscle cells, especially for the shifts from neonatal forms of myosin to the adult fast myosin heavy chain accumulation (King et al. 1987). Muscle development in embryonic and posthatch turkeys (embryonic day 18 to posthatch day 8) has been investigated in T_4 and goitrogen treated animals. The transition from embryonic to neonatal myosin heavy chain isoforms was blocked temporarily by either goitrogen (methimazole) or supplemental T_4 treatment but then occurred 6 days later despite continued treatment. These data indicate that thyroid hormones are involved in muscle myosin differentiation but are not absolutely required for it (Maruyama et al. 1995). The numbers of TR in different muscles during the development of young chickens is well correlated with the metabolic activity of the different muscle fiber types present (Dainat et al. 1984, 1986). Studies of domestic ducks treated with methimazole, T_4, T_3 or combinations of these treatments from 1-8 weeks of age showed cardiac and pectoralis muscle masses were decreased by 28% and 32% respectively, and body mass was decreased by 18%, compared to controls. This study also examined thyroid receptor gene expression and found TRα unaffected by methimazole treatment while TRβ 1 was decreased in leg muscles but not cardiac or pectoralis muscle. However, TRβ 1 expression was increased by thyroid hormone treatment in cardiac or pectoralis muscle (Bishop et al. 2000). Treatments that produce hypo- or hyperthyroid conditions in chicken embryos also can affect the proportion of fast and slow twitch fibers in the plantaris muscle of birds posthatch (at 3 or 35 days of age) and these effects were different in males and females (Dainat et al. 1991). These changes in the proportions of slow vs. fast muscle fiber types influence the aerobic capacity of locomotor muscles in adult ducks. Treatment with thyroid hormones for an 8-week period increased resting oxygen consumption and the activity of the aerobic enzyme citrate synthase in the left ventricle of the heart and in a leg muscle. However, there were no increases in muscle or body mass (Bishop et al. 1995). Studies of TRs in avian myoblast cultures indicate that the retinoic acid receptor (RXR) is important in the regulation of myoblast differentiation by T_3 (Cassar-Malek et al. 1996).

7.2.2.5 Gut

583. Thyroid hormones and glucocorticoids act together in gut maturation toward the end of incubation in chicken embryos. Glucocorticoids are involved in the maturation of intestinal glucose transport, thyroid hormones stimulate cellular differentiation and induce digestive enzyme production (Black and Moog 1978; Black 1988). More recent work has addressed the physiological details of the maturation of gut transport in birds (Obst and Diamond 1992). T_4 or T_4+cortisol *in ovo* stimulate precocious increases in glucose uptake in chick embryo intestine; cortisol alone does not have this effect. Similarly, organ culture studies of chick intestine show dose-dependent increases in some types of glucose transporters but there are some discrepancies between the *in vitro* and *in vivo* studies on this subject (review, Collie 1995). Studies of 5'D I in chick intestine suggest that intratissue deiodination may be important in generating T_3 for intestine development (Suvarna et al. 1993).

7.2.2.6 Lung

584. Thyroid hormones appear to be necessary for the maturational events preparatory to lung inflation in both birds (Wittmann et al. 1983) and mammals, with the timing of the effects differing in precocial and altricial species. However, the picture is complex and it is not clear whether thyroid hormones are acting directly on cell differentiation or proliferation. Thyroid hormones interact with glucocorticoids and prolactin in these lung maturation events and the relative roles of the different hormones and the nature of their interactions are not fully understood (review, McNabb 1992).

7.2.2.7 Liver Enzymes

585. In mammals and birds the control of malic enzyme and some other hepatic lipogenic enzymes is related to thyroid status, and this system has been used as an important model system for understanding the

mechanisms of thyroid hormone action. Typically hypothyroidism leads to a decrease in the mobilization and metabolism of lipids and to a lesser extent decreases in the synthesis of lipids. Hyperthyroidism also leads to increases in lipogenic activity.

586. Feeding by perihatch chicks is associated with rapid increases in both circulating T_3 and hepatic malic enzyme. Investigations of the molecular aspects of hormonal control of this system by T_3 have led to a number of discoveries about thyroid hormone action in birds. The regulation of malic enzyme synthesis is by both nutritional and hormonal factors and involves transcriptional effects (less important) and pretranslational effects (more important). In addition, the control of malic enzyme synthesis is tissue-specific (review, Goodridge et al. 1989).

587. Most of the work on the control of lipogenesis in birds has been with the goal of altering lipids in poultry production. Feeding T_3 decreases *in vitro* lipogenesis (IVL) at different protein levels in the feed although it did not compensate for some of the alterations caused by different levels of dietary protein (Rosebrough and McMurtry 2000). Hypothyroidism also can decrease IVL (as in mammals); T_3 replacement restores IVL initially but is followed by a decrease in IVL (Rosebrough and McMurtry 2003).

7.3 Role of Thyroid Hormones on Seasonal/Organismal Processes

7.3.1 Reproduction

588. In temperate latitude birds under natural photoperiods there is generally an inverse relationship between circulating reproductive steroids and thyroid hormones. Reproductive activities generally occur in the spring and summer when day lengths are increasing or long and stimulatory to breeding activities. Plasma thyroid hormones, which appear to be important in the initiation of gonadal development (section 7.3.1.1), decline during the early reproductive period. After some period of egg laying, the bird becomes refractory to the effects of long day length, reproduction ceases and postnuptial molting occurs. Thyroid hormones rise during the period when egg laying is declining and thyroid hormones (as well as prolactin) are thought to play a permissive role in the development of photorefractoriness (i.e. the lack of gonadal responsiveness to long days) because thyroidectomized birds do not become photorefractory. Thyroid hormones also are important in the molting process (section 7.3.4). This general seasonal picture applies to a number of wild bird species that have been investigated as well as domestic birds under natural photoperiods (Jallageas and Assenmacher 1979; Nicholls et al. 1988; Dawson 1989; Lien and Siopes 1993a). Thyroxine treatment results in testicular regression and decreased hypothalamic gonadotropin releasing hormone in male starlings, indicating that thyroxine mimics the effects of long day length (Boulakoud and Goldsmith 1991). The picture of thyroid hormone relationships in tropical birds is much more complicated (see below).

7.3.1.1 Gonadal Development

589. In mammals some cell types in both male and female gonads have TRs at least during development. There is relatively little information about whether thyroid hormones are required for the differentiation of gonads in either avian or mammalian embryos. At least part of this lack of information seems to have resulted from early work suggesting that gonads were unresponsive to thyroid hormones at least in the context of hormone-stimulated changes in oxygen consumption. This seems to have stifled research on the potential effects of thyroid hormones on gonads. However, there have been a number of cellular and molecular studies of the effects of thyroid hormones on mammalian testes (review, Jannini et al. 1995) and the detailed information from these studies suggests that similar experimentation is needed in both male and female birds.

590. Temporary exposure of both female and male chicks to thyroid inhibitors for several weeks affects later reproductive development and performance and manipulations of this type have been used in attempts to alter time of puberty, to attempt to improve reproductive performance in poultry and in reinitiating egg laying following molting (e.g. Peebles et al. 1994; Siopes 1997). It is generally accepted that thyroid hormones are involved in the onset of puberty in birds (Kirby et al. 1996). Recent studies have shown that in females hypothyroidism during embryonic chick development results in decreases in oocyte volume, nuclear size and mitochondria (Roda-Moreno et al. 2000). In male chicks thyroid hormone treatment for several weeks posthatch results in precocious puberty, larger testis size and increased sperm production but abnormal spermatogenesis (Kirby et al. 1996; Knowlton et al. 1999). These effects of transient hypothyroidism are similar to ones seen in developing rats in which TRs are now known to be present in Sertoli cells of developing testes but not in adult testes (review, Jannini et al. 1995). Testis size in rats is related to initial Sertoli cell numbers suggesting that thyroid hormones may be playing a role in testis size in birds as well (review, Thurston and Korn 2000).

591. In adult birds thyroid hormones are required for gonadal maturation; thyroidectomy prevents seasonal gonadal maturation in temperate latitude birds. In tropical and subtropical birds the picture is more complex with thyroidectomy resulting in a wide variety of responses in different species. A number of studies that have manipulated brain thyroid hormones in relation to photoperiod in American tree sparrows have addressed many of the interactions of T_4, T_3 and photoperiod on the programming of seasonal reproduction, photorefractoriness and postnuptial molt using a variety of experimental paradigms including intracerebroventicular injections of T_4 and T_3 (e.g., Reinert and Wilson 1997; Wilson and Reinert 1998 2000). The brain injection studies suggest that T_4 is more important than T_3 in its effects on the brain/HPT axis in the interaction of photoperiod and reproduction. A recent review of this literature as well as the historical literature on thyroid and photoperiod interactions in tropical birds suggests that thyroid hormones are playing an organizational role that influences the way in which gonadotropin releasing hormone neurons respond to photoperiod (Dawson and Thapliyal 2002).

7.3.1.2 Egg Production/Laying

592. Thyroid hormones are required for normal reproductive activity in female birds. Thyroid inhibition in adult hens is associated with decreased egg laying, and in the extreme, with complete cessation of egg laying in galliform birds (review, Decuypere et al. 1991). Hypothyroidism results in decreased egg production, egg weight, shell thickness and ovarian weight in poultry (Wentworth and Ringer 1986). Temporary treatment with goitrogens has been used as a strategy for altering the timing and performance of egg laying in chickens (Lien and Siopes 1993a,b). Treatment of developing female chickens with thiouracil from 0-6 or 6-16 weeks of age decreased plasma T_4, body weight and egg production. Some eggshell quality alterations were found in the experiment with thiouracil treatment from 6-16 weeks but these alterations did not occur consistently (Peebles et al. 1994).

7.3.2 Hatching

593. Historically, the observations that thyroid hormones peaked during the perihatch period and that thyroid inhibition interfered with hatching in chickens and quail led to the idea that T_3 was THE "hatching" hormone (Freeman 1974). General support for this idea came from the observation in precocial birds that the plasma T_3/T_4 ratio rises in conjunction with internal pipping into the air cell and remains high throughout the remainder of the perihatch period. Treatment of eggs with goitrogens late in incubation, and the resultant decrease in thyroid hormones, is associated with increased time between external pipping through the shell or failure to hatch, and sometimes with failure of yolk sac retraction in those embryos that do hatch (review Decuypere and Kühn 1988). However, the absence of a perihatch peak in all the altricial species studied to date (McNabb and Olson 1996) argues against thyroid hormones playing such an absolute role in hatching in all birds. In chickens, inhibition of thyroid function in hens decreases their egg

production, the hatchability of their eggs and embryonic mortality during the hatching process. Thyroid hormones stimulate a variety of metabolic and developmental processes necessary for successful hatching but the mechanisms by which these effects on hatching and survival are occurring are not fully understood. Goitrogen administration to eggs during incubation leads to failure in hatching and in yolk sac retraction. Early hatching is associated with increased plasma T_3 (review Decuypere and Kühn 1988). Small amounts of exogenous T_4 introduced into turkey eggs prior to incubation can improve hatchability (Christensen 1985) and differences in 5'D II activity and plasma T_4 concentrations (substrate availability) leading to T_3 production appear to play a role in different hatching times in weight-selected lines of chickens (McNabb et al. 1993).

7.3.3 Eggshell Formation

594. Many studies that have manipulated thyroid hormone availability (by goitrogens or iodine deficiency) have demonstrated that thyroid hormone deficiency decreases or eliminates (depending on the degree of deficiency) egg laying by hens and hatchability of those eggs that are laid (review, Decuypere et al. 1991). Temporary exposure to thyroid hormones can have some stimulatory effects on thyroid function during a rebound period after cessation of the thiouracil treatment and can stimulate the precocious onset of egg laying. Studies that exposed female chickens to thiouracil from 0-6 weeks and 6-16 weeks posthatch and followed egg production and egg quality for 28 and 38 weeks, respectively, found very complex interactions. The 0-6 week thiouracil treatment led to decreased body weight, egg weight and egg production from 20 to 28 weeks. The 6-16 week thiouracil treatment did not have these effects. Likewise, neither experiment gave evidence of associations between plasma T_4 and altered eggshell quality (Peebles et al. 1994).

7.3.4 Molt

595. The administration of thyroid hormones can induce molt and cessation of egg laying in birds as can feed restriction or a combination of these two approaches. It is common practice in the poultry industry to induce molt using various combinations of these treatments as well as manipulation of photoperiod and other dietary alterations (Decuypere and Verheyen 1986; Lien and Siopes 1993b). In seasonally reproducing wild birds and domestic birds that molt naturally, the cessation of reproductive activity and molt occur concurrently with increases in thyroid hormone (Goldsmith and Nicholls 1984a,b). A decrease in circulating estrogen also is associated with the initiation of molt (review, Decuypere and Verheyen 1986). Studies that have followed hormonal patterns during molt induced by feed restriction in chickens suggest that increases in both plasma T_4 and T_3 are associated with the induction of molt but that an increase in the thyroid hormone/estrogen ratios are associated with the induction of new feather papillae. Several studies suggest that T_4 is more important than T_3 in these processes (review Decuypere and Verheyen 1986). In turkeys, T_4 induces molt but T_3 does not (Queen et al. 1997). Other studies that have followed the patterns of hormones in molt induced by feed restriction argue that a decrease in progesterone is the key hormonal stimulus (i.e. primer) for the induction of molt and that T_4 is most important in feather regrowth (Herremans et al. 1988). Studies with T_4, T_3 and inhibitors of 5'D in tropical birds also provide evidence that T_4 is more effective in stimulating feather regeneration than T_3 (Kanchan and Chandola-Saklani 1995).

596. High plasma thyroid hormone concentrations, especially high T_4, are present in many species of wild birds during both the prenuptial and postnuptial molts, although the latter molt is much more extensive than the former (Assenmacher and Jallageas 1980). Molt is very energetically demanding both for new feather production and to balance the additional heat loss with poor insulation during the molt (review, Blem 2000) so it seems likely that the high thyroid hormones at this time are at least partially involved with the level of energy demand and thermogenesis.

7.3.4.1 New Feather Formation

597. Studies of the hormonal balances during molt in poultry suggest that estrogen decreases appear to be important in the initiation of molt but that new feather formation is promoted by an increase in the thyroid hormone:estrogen ratio. Detailed studies of thyroid hormones during the postnuptial molt in emperor and adelie penguins also show strong correlations between specific stages of feather replacement (initial growth of the new feathers and subsequent shedding of the old plumage) and thyroid hormones with plasma T_4 showing the closest relationships (Groscolas and Leloup 1986). A number of studies have suggested that T_4 directly affects the activity of feather papillae but it should be noted that these studies were done before the time when T_3 was recognized as the more metabolically active hormone so the results do not distinguish between the effects of T_4 and T_3 (review Decuypere and Verheyen 1986).

7.3.4.2 Feather Pigmentation

598. Because both thyroid and reproductive steroid hormones are important in molting, it is plausible that these hormones could affect feather pigmentation. However, it appears that most of the expression of feather color in males is part of the "neutral" developmental state and that less colorful female plumage results from estrogen suppression of the male color patterns. In a few species testosterone plays a role in male coloration (review, Owens and Short 1995). Thyroid hormone alterations affect a number of aspects of feather structure but there does not seem to be any published data linking feather pigmentation and thyroid hormones. An attempt to use feather pigmentation as a potential assay for thyroid disruption is discussed in Section 7.6.3.2.

7.3.5 Development and Maintenance of Photorefractoriness

599. There are differences in the roles that melatonin plays in birds and mammals. In mammals melatonin is involved in the coordination of reproduction with a favorable time of year. In contrast, in birds melatonin is involved in the entrainment of circadian activity rhythms. Melatonin also is involved in seasonal regulation of immune function and with the neuroplasticity of the avian song control system (Bentley 2001). The effects of melatonin are opposite in these two cases; melatonin enhances immune function but has an inhibitory effect on the song control system. Thus, during the breeding season, melatonin receptors are downregulated and this appears to occur through the same thyroid-dependent mechanism that controls reproductive state (Bentley 2001; section 7.3.1.1).

600. Relationships between the immune response and thyroid hormones have been suggested by some experiments in poultry. However, attempts to verify this in different lines of chickens found no relationship between thyroid hormones and antibody responses (Martin et al. 1988).

7.4 Role of Thyroid on Behavior

601. Although the roles of reproductive hormones in the different phases of reproductive behavior have received a great deal of study, thyroid hormones have not been considered in these studies. Thyroid hormones are involved in the neuronal regrowth in the vocal centers of the songbird brain (see section 7.2.2.1) but the behavioral implications of this hormonal role in development are not understood.

7.5 Role of Thyroid on Metabolism

7.5.1 Thermogenesis

602. Metabolic heat can be categorized into (1) essential heat, which is the metabolic heat produced by essential life processes and which is comparable in poikilothermic and homeothermic animals, (2) obligatory heat, which is the additional heat increment of resting heat production of homeotherms at

thermoneutral temperatures, and (3) regulatory or adaptive heat which is the extra heat produced by homeotherms in response to cool temperatures to maintain constant body temperature (Danforth and Burger 1984). Obligatory heat is generally considered to be directly under thyroid control, while regulatory heat historically has been considered to be primarily under nervous control with thyroid hormones playing a permissive role on factors such as tissue sensitivity to sympathetic nervous control or the capacity for heat production in thermogenic tissues (Danforth and Burger 1984). Some birds show increases in resting metabolic rate with acclimation to sustained cold (Dawson and Marsh 1989) so in these cases the capacity for sustained heat production in the cold alters obligatory heat production. A role for thyroid hormones in regulatory heat is indicated by the lack of thermoregulatory responses to cooling in hypothyroid chicks and adult birds (reviews Hillman et al. 1985; Jansky 1995).

603. Historically most avian thermogenesis in response to cold (regulatory heat) has been attributed to shivering of skeletal muscle. In contrast to mammals, birds do not possess brown adipose tissue in which oxidative phosphorylation is dissociated from the very high heat production in this tissue. The regulation of this dissociation is by an uncoupling protein (UCP). Searches for such a UCP in avian adipose tissue in cold acclimated birds and birds that undergo deep torpor have not been successful (review, Stevens 1996). However, an avian UCP has recently been identified in chicken and duckling muscle (Raimbault et al. 2001). Several lines of evidence suggest this UCP is playing a thermogenic role in birds. The expression (mRNA) of this avian UCP is increased in cold exposed chickens and ducks (Raimbault et al. 2001) and decreased in heat exposed chickens (Taouis et al. 2002). There are good correlations between increased heat production, increased avian UCP-mRNA and increased plasma T_3 concentrations in chicks exposed to cold for 7 days compared to chicks maintained at thermoneutral temperatures (Collin et al. 2003a). In addition it is regulated by T_3 (which stimulates thermogenesis), as indicated by increased UCP-mRNA in T_3 treated and decreased UCP-mRNA in goitrogen treated (methimazole or iopanoic acid) chickens (Collin et al. 2003b). Thyroid hormone effects on thermogenesis include both slow effects thought to be mediated through nuclear TR and more rapid effects that appear to affect mitochondria. Recently a form of the TRα has been identified in the inner mitochondrial membrane and a number of lines of evidence suggest this extranuclear pathway of action of T_3 may be involved in thermogenesis (review, Wrutniak-Cabello et al. 2001).

7.5.2 Development of Thermoregulation

604. Avian embryos initially produce only essential heat and the time of initiation of thermoregulatory responses differs in precocial and altricial birds. Precocial chicks show thermoregulatory responses during the perihatch period, altricials first showing thermoregulatory responses days or weeks after hatching. During early thermoregulatory development it is difficult to distinguish between obligatory and regulatory heat because this distinction is based on the animal having established a homeothermic resting metabolic rate. In the period when thermoregulation is developing, resting metabolic rate (obligatory heat) changes with increases in the stable body temperature the bird can maintain at a given age. As the bird's body mass increases (improved surface to volume ratio for heat conservation), its insulatory feather cover expands the length of the thermoneutral zone (the range of ambient temperatures where metabolic rate is basal). The longer the thermoneutral zone, the lower the critical temperature at which regulatory heat will be required, so older chicks have less need for regulatory heat production. Likewise, adult birds will require even less regulatory heat production in any given set of temperature conditions than will chicks. The development of thermoregulation in altricial and precocial chicks has been reviewed by Visser (1998) and the relationships between the development of thyroid function and the development of thermoregulation have been reviewed by McNabb and Olson (1996) and McNabb et al. (1998).

7.5.2.1 Precocial Species

605. The most detailed information linking thyroid and thermoregulatory development in birds is available for precocial galliform birds, chickens and quail. In these species, much of the increase in metabolic capacity occurs prehatch, i.e. it appears that the acquisition of obligatory heat production occurs during late incubation (review Vleck and Bucher 1998). Some regulatory heat production may be present in late embryonic life; chicken embryos near the end of incubation show an increased resistance to cooling or transient increases in heat production with cooling (review Visser 1998). Two studies suggest that these metabolic responses may be due to the increasing plasma thyroid hormones present at this time (section 7.1.4.1); 16.5-day chicken embryos increase plasma T_4 in response to cooling (Thommes et al. 1988), and chicken embryos treated with thiourea do not show the metabolic responses to cooling (Tazawa et al. 1989). However, it should be noted that in many studies precocial embryos have not shown metabolic responses to cooling (review Visser 1998). Precocial hatchlings characteristically show strong metabolic responses to cooling in association with the time of the perihatch peaks in plasma T_4 and T_3. Further evidence of the role of thyroid hormones in these thermogenic responses is that inhibition of thyroid function by goitrogens interferes with or eliminates these thermogenic responses (Freeman 1970, 1971). The increase in circulating T_3 and the T_3/T_4 ratio during the perihatch period is closely associated with the initiation of thermogenic responses at this time. These increases in circulating T_3 result from increased deiodination of T_4 to T_3 and decreased degradation of T_3 (section 7.1.4.6). Increases in hepatic T_4 to T_3 conversion are stimulated within hours after cold exposure in young chickens (3-5 weeks of age). These deiodinase responses appear to be inherent liver responses because they are independent of HPT and growth hormone axis control, i.e., they are present in thyroidectomized and hypophysectomized as well as control birds (Rudas and Pethes 1984, 1986). Thyroid function in precocial birds other than galliforms has not been studied in the context of the development of thermoregulation.

7.5.2.2 Altricial Species

606. Altricial embryos and early posthatch young are essentially poikilothermic, i.e. their body temperature approximates that of the environment and they do not show increases in metabolism in response to decreases in temperature. Altricial chicks develop thermoregulation at ages ranging from about 1 to 3 weeks posthatch depending on their "position" on the altricial-precocial spectrum (review, Visser 1998; Dawson and Whittow 2000). The pattern of circulating thyroid hormone development has been studied in several altricial species and is generally correlated with the pattern of thermoregulatory development (McNabb and Olson 1996). In brief, plasma thyroid hormones are extremely low in embryos and early posthatch life, then increase gradually to reach a plateau by the time the birds are attaining homeothermy (section 7.1.4.1). HPT axis maturation in altricial birds occurs posthatch, so the linking of the axis components involved in the relaying of a hypothalamic signal initiated by cold exposure is not present until several days after hatch (doves; McNichols and McNabb 1988). Essentially all of the studies linking thyroid function to thermoregulation in altricial species are correlative (review, McNabb and Olson 1996).

7.5.3 Lipogenesis

607. Thyroid hormone stimulates lipogenesis in mammals and the amplified response of malic enzyme, one of the lipogenic enzymes, is well studied in both mammals and birds (section 7.2.2.6). Studies of lipogenesis in relation to feeding regimes and T_3 effects in broiler chickens indicate complex responses of this system to thyroid hormone status and diet (e.g., Rosebrough 1999; Rosebrough and McMurtry 2003).

7.6 Assay Methods for Assessing Thyroid Disruption in Birds

7.6.1 Overview of Experimental Methods

608. The published studies on thyroid disruption in birds have used two categories of endpoints: (1) endocrine variables related to thyroid function or its HPT axis control and (2) target organ endpoints related to the developmental effects of thyroid hormones (McNabb 2005). This section will be organized according to those categories and will focus on the sensitivity of different methods for demonstrating thyroid disruption and its downstream effects. The presentation of the work will be in the context of known mechanisms of chemical action that depress thyroid function and that are consistent with the effects of these chemicals in well controlled laboratory tests. It should be noted that in a number of cases, studies have reported that some data on thyroid variables suggest increased thyroid function, especially at the low ranges of the chemicals used. These data are too limited to determine if these apparent stimulatory effects should be categorized as evidence of thyroid disruption or whether they reflect transient overshoots in circulating hormones as part of the compensatory responses of the HPT axis. Some examples of field studies will be cited, but in general field studies have not linked chemical exposure, thyroid alterations and other endpoints in a way that allows evaluation of the effectiveness of methods for thyroid disruption screening or testing.

7.6.2 Thyroid and HPT Axis Endpoints

7.6.2.1 Circulating T_4 and T_3

609. Measurement of circulating concentrations of thyroid hormones is the key indicator of whole body exposure and thus would seem to be the best measurement of organismal thyroid status. In human clinical medicine free-T_4 (fT_4) concentrations are used and they are typically supplemented by TSH measurements to provide information about HPT axis activation. Concentrations of fT_4 rather than total T_4 are preferred because the free fraction of hormone is considered to be that part available to tissues and because fT_4 concentrations are typically independent of hormone binding protein changes that occur in some physiological states. Some of the categorical problems/considerations that need to be taken into account in using hormone concentrations for assessing thyroid disruption in mammals have been discussed by DeVito et al. (1999). In birds, almost all of the available information is on total thyroid hormone concentrations.

610. In general, much of the data on circulating thyroid hormone concentrations in birds in relation to exposure to chemicals thought to alter thyroid function are highly variable and do not appear to give clear information about thyroid disruption. In rats, exposure to PCBs causes marked decreases in plasma T_4 and T_3 (see for e.g., Barter and Klaassen 1992a,b,1994). In contrast, a variety of laboratory and field studies in birds give much more equivocal and often inconsistent results (review, Scanes and McNabb 2003). Thus, in many of these studies one cannot tell whether the chemical is affecting thyroid function or whether the effect is obscured by variability in the circulating hormones. Studies of perchlorate exposure (perchlorate competitively inhibits thyroidal iodide uptake) in bobwhite quail have been used to compare the sensitivity of circulating thyroid hormone concentrations, thyroid gland weights and thyroid gland hormone content. These studies showed circulating hormones to be the most variable measurement and to be the least sensitive indicator of altered thyroid function (McNabb et al. 2004a,b). At the lower and mid range concentrations of perchlorate, both high and low hormone concentrations were observed in different individuals and from experiment to experiment. It seems likely that these variable responses reflect cyclic patterns of circulating hormone changes reflecting HPT axis responses. In brief, with initial exposure to perchlorate, plasma hormone concentrations will decrease, leading to increased TSH stimulation which in turn will increase hormone release from hormone stores in the gland, thereby restoring euthyroid levels of circulating hormone. The increased TSH also will stimulate thyroid gland growth and functional capacity

so at low perchlorate exposure levels this may partially compensate for the initial effects of perchlorate. However, if exposure continues, circulating hormone concentrations may again decrease and the cycle may repeat. This type of cyclic response pattern of circulating thyroid hormones, including times when circulating hormones overshoot in a positive direction, is known in iodine deficiency in humans (Delange and Ermans 1996) and in perchlorate-exposed rats (York et al. 2001). It seems likely that the large hormone stores in the thyroid, a feature unique to this endocrine gland, play an important role in setting up such cyclic patterns.

611. The use of fecal hormone assays to measure thyroid hormones has potential as a non-invasive technique for assessing thyroid disruption in some wild species, especially endangered ones. However, these assays are likely to be more variable than plasma measurements and in addition will have all the same potential problems (e.g. diurnal variations, food related differences, etc.).

7.6.2.2 Thyroid Mass and Histology

612. Historically, thyroid mass has been used as an indicator of HPT axis activation (resulting from low circulating thyroid hormones) in a number of contexts in avian endocrinology. In laboratory studies of ammonium perchlorate (bobwhite quail and mallard ducks) and in herring gulls exposed to PCBs in the field, thyroid mass and circulating T_4 are of approximately equal sensitivity for detecting decreases in thyroid function (McNabb et al. 2003; McNabb et al. 2004a,b; McNabb 2005).

613. Although alterations in thyroid histology, resulting from increased TSH stimulation associated with HPT axis activation, are regularly used as indicators of altered thyroid function in mammalian studies (DeVito et al. 1999), this type of assessment has received little use in birds. However, thyroid gland weight and histopathology were used to provide evidence of thyroid disruption in herring gulls collected from Great Lakes sites polluted with PCBs between the mid 1970s and the early 1990s (Moccia et al. 1986). Gulls at the high PCB sites had thyroidal microfollicular hyperplasia, as well as thyroid hypertrophy compared to gulls collected at the reference site in the Bay of Fundy. Although the lower iodine availability in the Great Lakes, compared to the marine environment of the reference site, could be a confounding factor in these studies, evidence from other studies suggests that this was not the key factor altering thyroid mass or histology (Moccia et al. 1986).

7.6.2.3 Thyroid Hormone Content

614. Thyroidal T_4 content measurements, which have not previously been used as an index of thyroid function, are a much more sensitive index of decreased thyroid function than either plasma thyroid hormones or thyroid gland weight in bobwhite quail chicks exposed to ammonium perchlorate for two weeks (McNabb et al. 2004a). In this study thyroidal hormone content revealed decreased thyroid function at ≥.05 ppm, thyroid gland weight indicated decreases at ≥500 ppm and plasma T_4 indicated decreases at ≥1,000 ppm. Preliminary studies with mallard ducks indicate the same ranking of these variables with respect to detecting thyroid function (McNabb et al. 2003) and studies of field caught herring gulls exposed to PCBs in the Great Lakes also support this pattern (McNabb and Fox 2003).

7.6.2.4 Deiodinase Activity

615. Exposure to commercial PCB mixtures (Aroclors) decreases hepatic 5'D I activity and increases brain 5'D II activity in laboratory rats (Morse et al. 1993, 1996; Raasmaja et al. 1996). In chicken embryos from eggs dosed with 6.7 ppm Aroclor 1242, hepatic 5'D I activity just before hatch was significantly decreased; Aroclor 1254 at the same dose did not alter hepatic 5'D I (Gould et al. 1999). Brain 5'D II activity in herring gulls exposed to PCBs in the Great Lakes showed no relationship to site PCB exposure (Fowler 2001). Thus, based on these two studies in birds, hepatic 5'D I activity may have promise for

detecting thyroid disruption. Tests of the responsiveness of brain 5'D II in relation to a different level of thyroid disruption need to be performed.

7.6.2.5 Transthyretin and Free Thyroid Hormone Concentrations

616. Some chemicals, e.g. certain hydroxylated PCBs competitively displace T_4 from mammalian TTR *in vitro* (McKinney et al. 1985; Cheek et al. 1999) and there is some evidence that this is important in decreasing thyroid function in rats (Brouwer and Van den Berg 1986). However, in birds, transthyretin also binds T_3 and with higher affinity than T_4 (Chang et al. 1999). Recently this technique has been used to compare the inhibition, by a number of medical, industrial and agricultural chemicals, of T_3 and rT_3 binding to chicken TTR. Most of the chemicals tested (e.g., diethylstilbestrol, pentachlorophenol, ioxynil, dicofol) were effective in competitively displacing T_3 binding from TTR although dicofol showed a biphasic effect, inhibiting T_3 binding at some concentrations and facilitating binding at others (Ishihara et al. 2003). This method also has been used to test for inhibition of T_3 binding to chicken TTR by bisphenol A, nonylphenol and seven chlorinated derivatives of these compounds found in effluents from paper manufacturing plants. Biphenol A and nonylphenol were the most effective competitive inhibitors of T_3 binding, and less chlorinated derivatives were poorer competitors than more highly chlorinated ones (Yamauchi et al. 2003).

617. When chemicals competitively displace T_4 from TTR binding, presumably this T_4 displacement should increase circulating fT_4 concentrations thereby enhancing T_4 metabolism and excretion. Herring gull plasma from birds at high PCB sites showed a trend toward higher fT_4 concentrations than at the reference site. Surprisingly plasma fT_3 was not altered in relation to PCB exposure (Maher et al. 2002, 2003). These data are suggestive of PCB displacement of T_4 from transthyretin at high PCB sites. The opposite pattern was seen in cormorants from a western European site with approximately 2-fold higher PHAHs than the reference site; fT_4 was significantly decreased in these birds (Van den Berg et al. 1994). In contrast, common tern hatchlings from eggs with a 6.4X range of site PCBs showed no significant differences between sites in plasma fT_4 (Murk et al. 1994). Overall, these correlative studies are difficult to interpret and are not convincing that fT_4 measurements are likely to be an effective indicator of thyroid disruption.

7.6.2.6 Receptor Binding Assays and Thyroid Receptor Expression

618. Chemicals that disrupt reproductive endocrine function often act by binding to estrogen or androgen receptors and act as hormone agonists or antagonists. Thus for assessing chemical potential for reproductive disruption, receptor binding and expression assays are powerful tools. In contrast, very few chemicals bind to either mammalian TR (Cheek et al. 1999) or avian TR (Ishihara et al. 2003; Yamauchi et al. 2003) so this technique has little relevance for screening chemicals for thyroid disruption.

619. Thyroid receptor binding and receptor expression assays do have utility in basic research on the effects of chemicals. Altered thyroid function is likely to result in receptor up or down regulation in tissues and this may be an important part of the nature of the response to the chemical. Studies of TR cited in sections 7.1.4.6, 7.1.4.7 and 7.2 indicate ways in which these assays could be used to understand the effects of chemicals on thyroid function and thyroid effects on target organs.

7.6.2.7 Altered Hormone Excretion

620. Increased T_4 excretion resulting from the induction of hepatic UDP-GT (which glucuronidates T_4 and facilitates its excretion in mammals) is an example of an indirect mechanism that alters thyroid function. Thus, UDP-GT activity could be used as a marker of exposure to certain chemicals with the potential for thyroid disruption (section 7.1.4.8). McCleary (2001) measured UDP-GT in chicken embryos

exposed to PCB 126 *in ovo*. However, although there were trends toward decreased hepatic UDP-GT, thyroid function changed little so these studies do not adequately address the usefulness of the technique in assessing thyroid function. Murk et al. (1994) measured hepatic UDP-GT in terns exposed to mixed PHAHs in the environment. They found no significant differences in UDP-GT and no differences in the thyroid variables used in relation to PCB exposures. Thus to date there are no adequate data, based on relationships between UDP-GT activity and differences in thyroid state, for evaluating this type of assay for assessing thyroid disruption in birds.

7.6.3 Target Organ Endpoints

7.6.3.1 Growth Measures

621. Because thyroid hormones are required for growth in birds (section 7.2.1), body and tissue growth are generally thought to be suitable endpoints for assessing the effects of thyroid disruption. Several studies have measured growth in relation to thyroid disruption in birds exposed to contaminant chemicals in well controlled experiments (see below). Overall, neither hindlimb growth nor body weight seem promising as sensitive indicators of thyroid disruption in precocial embryos or chicks.

622. Embryos: Gould et al. (1997) studied the effects of Aroclor 1242, Aroclor 1254, or PCBs 54, 77 or 80 introduced into eggs and measured pituitary GH content, body mass, femur length and thyroid hormones in chicken embryos from those eggs on day 17 of the 21-day incubation period. They also measured plasma GH and IGF but found no effects of any treatment on these growth-related hormones. If body weight and femur length are useful indicators of decreased thyroid function, then they should be positively associated with decreases in thyroid function. At the highest dose of Aroclor 1242 (6.7 ppm) both plasma T_4 and body weight tended to be decreased and femur length was significantly decreased. The highest dose of Aroclor 1254 (6.7 ppm) did not affect thyroid hormones yet both body weight and femur length were significantly decreased. One individual PCB congener (PCB 77) significantly decreased body weight and femur length, significantly increased GH and tended to decrease plasma T_4, but these effects were only at one intermediate dose and there was no consistency to the trends seen at other doses. The other individual congeners used did not show any consistent pattern of effects.

623. In a second study, Gould et al. (1999) used the same PCBs and doses but the chicken embryos were sampled at the end of incubation (day 21) when 5'D I activity was high. In this study, the 6.7 ppm dose of Aroclor 1242 significantly decreased plasma T_4, plasma T_3 , 5'D, femur length and relative liver weight but not body weight. Aroclor 1254 (6.7 ppm) decreased plasma T_4 and femur length but not plasma T_3, relative liver weight or body weight. None of the individual congeners showed any consistent pattern of effects. Overall these studies show that decreases in femur length are positively associated with decreases in plasma T_4 in some but not all cases.

624. Chicks: Studies of the effects of ammonium perchlorate (AP) on thyroid function and growth in bobwhite quail chicks also provide evidence that growth is a relatively insensitive indicator of thyroid disruption. In dose response studies with quail chicks exposed for 8 weeks (beginning a few days after hatch) to a series of AP concentrations up to 4,000 ppm in drinking water, body weight was unaffected and femur and tibia growth were decreased significantly only at the highest (4,000 ppm) AP concentration used. Thyroid gland hypertrophy at concentrations ≥ 1,000 ppm, decreased plasma T_4 at ≥ 2000 ppm and decreased thyroidal hormone content at ≥. 0.05 ppm suggested that growth-related variables are much less sensitive indicators than all other measures of thyroid function that have been tested in birds. It appears that sustained periods of thyroid deficiency are required before body and skeletal growth are affected in these young precocial birds (McNabb et al. 2004a).

7.6.3.2 Feather Characteristics

625. Thyroid hormones are critical to feather replacement and feather abnormalities are associated with altered thyroid function. In addition, changes in estrogen and in the thyroid:estrogen hormone ratios play a role in the timing of molt (section 7.3.4). Thus, since PCB exposure has sometimes been associated with decreased thyroid function, molt and plumage characteristics might be a suitable endpoint for assessing downstream effects of PCBs. Quinn et al. (2002) tested this idea in American kestrels exposed to Aroclor 1242. The highest dose used was one reported to disrupt reproduction, the birds were dosed from the beginning of egg laying until the postnuptial molt was complete (6 months) and plasma was collected for hormone analyses weekly for 5 weeks postmolt. There were no effects of the Aroclor 1242 treatment on plumage color or reflectance or on plasma estradiol concentrations. In females, plasma T_4 was significantly decreased at the highest dose compared to controls at only one of the 5 weeks. In males, there were no significant changes in plasma T_4 but concentrations tended to decrease. Unfortunately the lack of significant, consistent thyroid hormone effects from the PCB treatment in this study do not allow any judgments of the usefulness of feather characteristics as an indicator of thyroid disruption.

7.6.3.3 Expression of Target Organ Developmental Genes

626. Alterations in the expression of target organ genes or translation of their proteins that are under the control of thyroid hormones, when correlated with known anatomical, physiological or metabolic effects, can provide information about the consequences of thyroid disruption. Currently, such endpoints in the developing central nervous system are the focus of a great deal of research (Chapter 4) in mammals but have received little if any attention in birds.

7.6.3.4 Tissue Differentiation in Target Organs

627. Avian embryonic pelvic cartilages, in which cartilage differentiation events are known to be responsive to T_3, may be a useful model for evaluating target organ effects of thyroid disruption (section 7.2.2.2). Likewise, the techniques that have recently been used to follow neuronal turnover, cell differentiation and apoptosis in relation to hormone effects in the vocal center regions of songbird brains (Tekumalla et al. 2002; section 7.2.2.1) seem to be potentially useful techniques for determining how thyroid disruption alters brain development in young animals. However, techniques of these types have not been utilized in the context of chemically induced thyroid disruption.

7.6.3.5 Brain Morphometrics

628. Exposure to dioxins and dioxin-like compounds is associated with the development of grossly asymmetrical brains in domestic and wild bird species (Henshel et al. 1997a,b; Henshel 1998; section 7.2.2.1). It seems likely that thyroid disruption is playing a role in this effect although measurements of thyroid function were not made in these studies. Evaluation of the relationships between thyroid disruption and brain morphometry should be evaluated as possible methods for the assessment of thyroid disruption effects on a key target organ, the brain.

7.6.3.6 Behavioral Tests

629. Although alterations in specific reproductive behaviors may have merit for revealing disruption of reproductive hormones, no specific set of behaviors linked to thyroid function are known. Thus, behavioral alterations associated with thyroid disruption are likely to be non-specific effects resulting from altered brain architecture and function during development. A number of behavioral tests have been used in mammals (DeVito et al. 1999) but not in birds. One specific area that might be promising is behaviors related to hearing (or direct tests of hearing) because thyroid deficiencies are known to permanently alter the development of hearing in mammals (Goldey et al. 1995a,b; 1996a,b).

7.6.3.7 Photorefractory Induction/Maintenance

630. Although thyroid hormones are important in photorefractoriness, there are variations in the responses seen in different species, differences between temperate vs. tropical birds, and delays in timing between thyroid effects on the higher parts of the hypothalamic-pituitary-gonadal axis and reproductive system effects (section 7.3.1.1). In addition, thyroid hormones are interacting with gonadal steroids and other hormones in their effects on reproductive timing. Thus, the complexity of this system argues against it being useful in assessing thyroid disruption.

7.6.4 Recommended Assay Protocols

7.6.4.1 The Avian Two-Generation Toxicity Assay (EPA DRP Contract # 68-W-01-023; 2003)

631. This includes a number of thyroid-relevant endpoints that will be measured in parental or subsequent generations of birds. The exposure of hens (parental generation) to chemicals will ensure that if transfer of the chemical to eggs occurs, embryos will be exposed *in ovo.* Secondly, the exposure of half of the F1 chicks to the chemical will allow separation of pre- and posthatching effects. The thyroid-relevant endpoints in this assay are listed in Table 7-1 below. The first four endpoints (plasma/serum thyroid hormones, plasma/serum TSH, thyroid weight, and thyroid histology) are intended to detect thyroid disruption, and the next two (bone length measurements and skeletal X-rays) are addressed to target organ effects of altered thyroid function. The final endpoint listed (plasma steroids) presumes a relationship between plasma/serum steroid and thyroid hormones. However, such a relationship is not documented in the avian literature. The two endpoints listed in the Avian Two-Generation assay that are most promising with respect to sensitive detection of thyroid alterations are plasma/serum TSH and thyroid histology. However, antibodies are not available for measuring TSH in birds. Thyroid histology procedures for avian studies have not been standardized, nor has the sensitivity of this endpoint been investigated. Thyroid histology is a relatively-to-very sensitive endpoint for detecting HPT axis activation in mammals, so it is likely to be of similar sensitivity in birds.

632. The second section of Table 7-1 addresses other endpoints that have been used or suggested in other documents to be useful in these assays. Based on the available information, thyroid gland hormone content is the most promising because it is very sensitive to altered thyroid function (section 7.6.2.3) and it is less labor-intensive than other potentially sensitive endpoints such as histological evaluations. The other endpoints listed in this section of the table are either very insensitive or they require considerable development to determine their sensitivity and practicality.

633. Endpoints that measure the effects of thyroid alterations on target organ endpoints have been very limited and mostly confined to indicators of growth. In general these endpoints are useful only if birds have experienced sustained organismal hypothyroidism (section 7.6.3.1). Biochemical and molecular techniques that measure endpoints associated with the effects of thyroid alterations on differentiation offer promise, but these techniques have not been developed for avian assays. Behavioral assays also offer promise as noninvasive techniques that do not require animal sacrifice. However, there is no available information about the efficacy or sensitivity of these techniques.

634. A wide variety of possible endpoints are listed in Table 7-2 below. To date, the most sensitive endpoint for detecting altered thyroid function in birds is the measurement of thyroid gland hormone content, of which >95% is T_4 (sections 7.6.2.1 through 7.6.2.3). This measurement capitalizes on a unique feature of thyroid glands, namely their capacity to store large amounts of thyroid hormone. This measurement detects alterations in function before changes are severe enough to alter organismal-level thyroid function (i.e., before the body as a whole is exposed to sustained decreases in plasma thyroid hormones). In addition, the measurement of thyroidal hormone content is less labor-intensive and more

easily quantified than histological assessments, which, based on studies in mammals, also may be very sensitive endpoints.

635. Thyroid gland weights are the second most sensitive assay currently available for birds, at least until plasma TSH assays are developed. Plasma thyroid hormone measurements are very insensitive indicators of altered thyroid function. Plasma hormone concentrations are highly variable, probably because of cyclic patterns of HPT axis responses as the system attempts to compensate for alterations in thyroid function partly by the release of thyroidal hormone stores and partly by increased gland function (section 7.6.2.1).

636. As mentioned above, at present there are no measurements/assays of downstream target organ effects that have been shown to accurately reflect the final effects of thyroid disruption in birds. Body and hindlimb growth measurements are very insensitive. Differentiation processes appear to be promising candidates for the development of endpoint measurements, e.g., cartilage to bone differentiation processes in pelvic cartilages from avian embryos have been shown to respond to T_3, and there are a number of ways in which their differentiation could be assessed. Molecular and cellular differentiation events in central nervous system, heart, or skeletal muscle development also have promise. The investigations in these latter areas are developing more quickly in mammalian studies than in birds, but it appears that many of these events are sufficiently alike in birds and mammals that the same assays can be used in both.

637. In summary, the sensitivity of the Avian Two-Generation Assay for detecting thyroid disruption in birds could be improved by the addition of measurements of thyroid gland hormone content. Comparative studies of thyroid gland hormone content and thyroid histology are needed to determine whether histology is more or less sensitive than measurements of thyroid gland hormone content. With respect to the effects of thyroid disruption on target organs, there are currently no sensitive assays that have been developed for use in birds. Biochemical or molecular endpoints indicative of alterations in target tissue differentiation seem most promising for the development of new assays.

7.6.4.2 Embryo One-Generation Assay

638. Measurements of a variety of reproductive endpoints in hatchlings exposed to contaminants *in ovo* have been proposed as screening and testing assays. Studies of the effects of estradiol and other potential reproductive toxicants in this type of assay are currently in progress (personal communication, M.A. Ottinger). In these studies the chemical is introduced into the egg early in incubation, half of the hatchlings are sampled and the remaining half are reared and sampled at a later time. The endpoints to be measured are indicators of reproductive endocrine disruption. However, the effects of chemicals on the development of thyroid function could be evaluated concurrently by the addition of measurements of thyroid gland hormone content or thyroid histology (see discussion above). The other endpoints commonly used to reflect altered thyroid function probably would not be useful unless embryos and chicks were sampled at several stages because subtle differences in maturation can markedly increase endpoint variability when only a single sampling time is used. For precocial species (Japanese or bobwhite quail, or mallard ducks) thyroid function undergoes considerable maturation during embryonic life, so sampling either prior to the perihatch period or shortly after hatching should allow effective detection of thyroid disruption. In contrast, altricial birds have little thyroid development until some time after hatch so they would need to be sampled later in development. The available literature is not adequate for evaluating whether this type of embryo exposure assay would be more sensitive for screening or testing than the measurement of thyroid endpoints within the Two-Generation Assay. However, an evaluation of embryo assays seems warranted because they could potentially require relatively short studies for thyroid screening.

Table 7-1 Existing or Potential Assays in Birds

Assay Name	Species	Major Thyroid-Related Endpoints	Target Effects Relevant to the Thyroid System	Status of Assay	
				Advantages	Disadvantages
Avian Two-Generation Assay	Bobwhite or Japanese quail, Mallard ducks	Plasma/serum T_4 and T_3	Organismal level thyroid function may affect differentiation of target organs/tissues including CNS, growth	Doesn't require sacrifice. Relatively inexpensive, easily validated.	Plasma T_4 and T_3, highly variable due to cyclic, compensatory responses.
		Plasma/serum TSH	HPT axis activation resulting from feedback of altered thyroid hormones.	Doesn't require sacrifice. Relatively inexpensive, easily validated.	TSH assays not available.
		Thyroid weight	HPT axis activation resulting from feedback of altered thyroid hormones.	Simple, quick, inexpensive.	Relatively insensitive, variation in dissection, requires sacrifice.
		Thyroid histology	HPT axis activation resulting from feedback of altered thyroid hormones.	Potentially very sensitive but details of sensitivity have not been documented.	Relatively labor intensive, has received little use in avian thyroid studies.
		Bone length measurements	Target organ effects of decreased thyroid function during skeletal development.	Simple, quick, inexpensive.	Not well investigated. Variable results from different studies. Difficult to standardize measurement techniques.
		Skeletal X-rays	Target organ effects of decreased thyroid function during skeletal development.	Should reduce variability in measurements.	Has not been used in published studies.
		Plasma steroids	Undefined relationship.	Doesn't require sacrifice, relatively inexpensive, easily validated.	No systematic investigations indicating relationship to thyroid function.

Assay Name	Species	Major Thyroid-Related Endpoints	Target Effects Relevant to the Thyroid System	Status of Assay	
				Advantages	Disadvantages
Other endpoint measurements not included in current assays	Bobwhite or Japanese quail, Mallard ducks.	Thyroid gland hormone content of T_4 and T_3.	Depletion of gland hormone stores due to release to maintain euthyroid circulating levels.	Very sensitive, effects appear early and at lower exposure levels than revealed by other methods. Has not been validated but validation should be straightforward	Requires sacrifice.
		Thyroid peroxidase.	HPT axis activation resulting from feedback of altered thyroid hormones.	Potentially sensitive. Validation should be straightforward.	Requires sacrifice. No avian assays available.
		Body weight	Indirect thyroid hormone effects on target organs.	Very easy, very inexpensive. Doesn't require sacrifice.	Extremely insensitive.
		Neurobehavioral tests	Thyroid hormone effects on nervous system development.	Potentially sensitive, doesn't require sacrifice.	No avian assays have been tested.
		Cold stress tests	Thyroid hormone effects on thermoregulatory ability.	Likely to be very insensitive. Doesn't require sacrifice.	No avian assays have been tested.

Table 7-2 Points of Thyroid Disruption in Birds

Disruption or Evaluation Site	Endpoints of Interest	Target Effects of Disruption	Assay Availability	Status of Assay
Organismal thyroid status: circulating hormones	Serum/plasma T_4, T_3	Organismal thyroid status affecting all target organs/tissues (developmental and metabolic effects);	Yes	RIAs and ELISAs; in common use.
HPT axis activation	Serum/plasma TSH	Reflects HPT axis feedback effects from altered serum/plasma T_4 and T_3 due to alterations in thyroid gland function or hormone turnover.	No	No available avian antibodies; heterologous antibodies don't cross-react.
HPT axis activation	Thyroid gland weight	Reflects HPT axis feedback effects from altered serum/plasma T_4 and T_3 due to alterations in thyroid gland function or hormone turnover.	Yes	Requires some training; consistent trimming of glands during dissection.
HPT axis activation	Thyroid gland histology	Reflects HPT axis feedback effects from altered serum/plasma T_4 and T_3 due to alterations in thyroid gland function or hormone turnover.	Yes	Not validated/standardized as a technique for avian thyroid studies.
Thyroid gland: iodide uptake	Na-I symporter	Decreased thyroid gland uptake of iodide resulting in decreased hormone synthesis.	Yes	Radioiodide uptake.
Thyroid gland: hormone content	Thyroidal T_4 and T_3 content	Altered thyroid hormone stores. Sensitive to release of stored hormones to maintain serum/plasma concentrations when hormone synthesis or turnover is decreased.	Yes	Used in avian thyroid research. Not validated across different laboratories.
Thyroid gland: TPO	TPO	Altered thyroid peroxidase activity; effects on thyroid hormone synthesis.	Yes	Has not been used or validated for avian thyroid studies.
Target tissues: hormone supply	Binding protein effects on free hormones	Enhanced free hormone concentrations (and consequent increased hormone turnover) resulting from competitive displacement of hormones from binding proteins by contaminant chemicals.	No	Assays could be developed, but may have limited utility sensitivity.
Target tissues: receptor binding	T_3 receptors	Altered hormone action. Few chemicals bind to thyroid receptors so these measurements are of minor relevance to thyroid disruption.	Yes	Receptor binding assays.
Target tissues: growth	Body or organ growth	Altered body or organ weights or skeletal dimensions.	Yes	Requires some training for consistency of measurement.

Disruption or Evaluation Site	Endpoints of Interest	Target Effects of Disruption	Assay Availability	Status of Assay
Target tissues: brain morphometrics	Brain morphometrics	Altered development of brain regions.	Yes	Not definitively linked to thyroid effects; could be reflecting direct effects on CNS.
Target tissues: development	Markers of tissue differentiation.	Altered tissue differentiation in central nervous system, muscle or skeletal tissues. Could include gene expression assays.	No	Assays used in mammals, have not been used in birds.
Target tissues: metabolism	Metabolism (oxygen consumption)	Altered metabolism in adults or altered thermoregulatory development in young birds.	Yes	Likely to be too insensitive to be useful.
Target tissues: deiodinases	Brain 5'D II activity	Central nervous system regulation of tissue T_3 concentrations may reflect responses to alterations in circulating thyroid hormone concentrations.	Yes	Sensitivity unknown for use as a screening tool.
Target tissues: molting and feather characteristics	Altered feather replacement	Disruption of hormonal control of the molting process.	No	Limited studies available suggest assays would be of limited utility and low sensitivity.
Target tissues: behavior	Altered sensory and motor performance	Altered central nervous system development reflected in behavior.	No	Tests of behaviors dependent on sensory or motor functions known to be thyroid responsive during development.
Thyroid hormone excretion	Phase II liver transformation enzymes; UDP-GT	Increased T_4 excretion resulting from contaminant induction of uridine diphosphate glucuronosyltransferase activity, i.e. increase in T_4 glucuronidation which enhances excretion in bile.	Yes	Assays not validated for evaluating contaminant effects, seem unlikely to be sensitive.

8.0 SUMMARY AND IMPLICATIONS

639. Thyroid hormones are essential for normal development in mammals, birds, amphibians, and fishes. Therefore, chemicals in the environment that interfere with the ability of thyroid hormones to play their normal role in development could have devastating effects on wildlife or human populations, and on individuals that make up those populations. Considering the role of thyroid hormones in development, it is important to construct screens and tests for potential thyroid toxicants in any endocrine disrupter screening and testing program. These screens and tests should adequately capture the range of points within the thyroid endocrine system that may be disrupted by these toxicants. A central goal of this DRP is to review the current literature on thyroid endocrinology in mammals, birds, amphibians, and fish; to review and evaluate current screens and tests under consideration by various committees charged with developing a comprehensive battery that will evaluate chemicals for thyroid disruption within the context of this literature (see Table 8-1 below); and to make recommendations to consider additional assays or endpoints that address specific weaknesses in the current assays.

640. Several important features of the thyroid system are conserved across all taxa. The structure of T_4 and T_3 is the same in all taxa, as is the mechanism by which they are synthesized. Moreover, T_4 is the principal hormone secreted from the thyroid gland, and T_3 is the most hormonally active form in the tissue. Peripheral conversion of T_4 to T_3 contributes to controlling tissue sensitivity to thyroid hormones in all vertebrates. Thus, blood levels of T_4 represent a measure of thyroid function, and blood levels of T_3 represent a measure of peripheral deiodination of T_4. Because some animals are very small (e.g., amphibian larvae, flounder larvae), it may not always be practical to measure blood levels of hormones. Therefore, it may be necessary to develop and validate methods that utilize tissue for hormone measurements.

641. The functional interactions among levels of the HPT axis also are similar, though not identical, among vertebrates. The hypothalamus controls the pituitary, which controls the thyroid gland. Negative feedback of thyroid hormones controls the hypothalamic-pituitary axis. However, in amphibians – at least during metamorphosis – the hypothalamic peptide responsible for pituitary-thyroid activity is not the same as in other vertebrates. Thus, while the general functionality of the system is the same among the vertebrates, there are differences in specific molecules that must be considered.

642. Thyroid hormone does not regulate the same developmental or physiological endpoints in all organs within a single animal, and the same is true across all vertebrates. Thus, thyroid hormones control events in the metamorphosing amphibian that are likely to be different in human development. However, within the context of thyroid toxicology, these different endpoints can be viewed as ways of testing the hypothesis that a specific chemical can interfere with thyroid hormone action. For example, the drug propylthiouracil (PTU) can reduce blood levels of thyroid hormone in both amphibians and in rodents. However, PTU-induced reductions in blood levels of thyroid hormone will not affect the same endpoints in the two species, but will similarly be indicative of an antithyroid agent.

643. All known thyroid toxicants have been identified by their ability to alter serum levels of thyroid hormones (Brucker-Davis 1998) because this is currently the only definition of thyroid toxicity. It has been reasonably argued that serum concentrations of thyroid hormones should be an indicator of all thyroid toxicants (DeVito et al. 1999). Hormone levels will reveal thyroid toxicants that interfere with thyroid function (by any mechanism), thyroid hormone metabolism (by any mechanism), or TR activation (in principle). For example, chemicals that inhibit thyroperoxidase would reduce T_4 synthesis and would suppress serum T_4. Likewise, chemicals that increase thyroid hormone metabolism and clearance from serum (e.g., UDPGT inducers) would cause a reduction in serum T_4 or at least an increase in serum TSH

(to maintain normal T_4 levels). Finally, chemicals that interfere with TR activation should alter the negative feedback action of thyroid hormone at the hypothalamus and pituitary, thereby causing a change in serum thyroid hormone levels. Thus, hormone levels are and will remain important indicators of thyroid toxicity.

644. However, changes in serum hormone concentrations do not indicate the specific effects that these changes will have on an organism. Thus, while a strong argument can be made for using serum hormone concentrations and thyroid weight/histology as the sole indicators of thyroid toxicity, their value in risk assessment is complicated because not all toxicants produce changes in serum T_3, T_4, TSH and thyroid histology that are consistent with the idealized model of thyroid physiology based on the effects of PTU (Zoeller 2005). Thus, some toxicants will produce dose-responses that do not follow this idealized model (see section 4.17), and there will be confusion about when changes in these endpoints leads to "compensation" versus a clearly harmful effect.

645. As reviewed in this document, new research indicates that endpoints can be developed that will likely prove to be sensitive indicators of adverse effects of thyroid hormone insufficiency and of thyroid toxicity. These endpoints represent measures of thyroid hormone action, both in development and in the adult. While we await development of new measures for these assays by the scientific community, changes can be made immediately to improve the sensitivity of the current assays. For example, alterations in thyroid hormone levels during the early postnatal period are currently not accounted for in any of the existing assays; these measurements should be incorporated into the screens. Specifically, T_4 levels in normal rat pups are in the range of 0.5 to 1.0 µg/dL on PND 4 (Goldey et al. 1995; Zoeller et al. 2000), rising to 8 to 12 µg/dL on PND 15, then declining to adult levels of approximately 3 µg/dL by PND 21. Thus, chemicals that affect serum hormone levels on P15, but not on P21, would not be captured in an experimental protocol in which P21 was the only time that serum thyroid hormone levels were measured. Incidentally, the radioimmunoassay used extensively in toxicological research is a commercial kit based on human serum and calibrated for human serum T_4 levels that are slightly higher than for rats. This kit has a lowest standard of 1 (or in some kits 2) µg/dL. Because serum samples that do not have T_4 levels above that of the lowest standard cannot be interpreted, measurements in the literature should be carefully evaluated because many of these are below the detectability of the assay kit used. Moreover, although the structures of thyroid hormones (T_4 and T_3) are identical among all vertebrates, the composition of the serum differs among animals, which may confound the assay.

646. Finally, the combined use of *in vivo* and *in vitro* screens for thyroid toxicants requires careful consideration. The number of targets of thyroid toxicity are numerous – in the thyroid gland alone, toxicants are known to interact directly with the sodium-iodide symporter, the transport protein Pendrin, the peroxidase enzyme, and enzymes of thyroglobulin catabolism. Each of these points of disruption produces a slightly different dose-response effect on serum hormone levels. In addition, thyroid hormone synthesis depends on the dual oxidase enzymes for the local production of hydrogen peroxide and there may be environmental chemicals that interact with these proteins. There is evidence that TSH signalling in the thyroid gland can be regulated by iodocompounds that may also be targets of disruption. Finally, once thyroid hormone is released into the blood stream, a large number of factors can influence its ability to play its role in development and physiology. Serum binding proteins are known targets of toxicants, though it remains unclear if these effects mediate observed actions of these toxicants. Likewise, toxicants can induce metabolizing enzymes in the liver (UDPGTs). However, for thyroid hormone to gain access into cells, they must interact with specific transporters, the OATPs and MTC8 proteins. These are clearly physiologically important, but we have no knowledge of their vulnerability to specific toxicants. And finally, T_4 must be converted to T_3 to exert an effect on the thyroid hormone receptor. This large number of regulatory points is difficult to imagine being incorporated into a battery of *in vitro* screens. Perhaps more importantly, even if each one of these steps could be separately evaluated in an *in vitro* assay, given the limited knowledge we have at this time one each aspect of the thyroid system, only an *in vivo* assay

could monitor the way these points of regulation respond to a perturbation by toxicant exposure. For example, we might easily show that a low dose of toxicant exposure can inhibit the TPO enzyme. But, to what extent must TPO be inhibited before thyroid hormone synthesis is inhibited. Moreover, to what extent must thyroid hormone synthesis be inhibited before circulating levels of thyroid hormone are compromised? And finally, to what extent must thyroid hormone levels be reduced (or increased) and for what duration, before an adverse outcome can be predicted? Thus, considering the complexity of this system, it is highly unlikely that *in vitro* assays can replace *in vivo* screens for the foreseeable future. Still, *in vitro* assays may well be employed, with caution, to identify or eliminate specific mechanisms of action.

647. Considering the biology of thyroid hormone action in development, a number of conclusions can be made regarding our ability to develop a cogent battery of screens and tests that would effectively evaluate chemicals for the ability to interfere with thyroid hormone signalling. These conclusions are presented below, but the reader is strongly encouraged to refer to the background information presented in this document used in making these conclusions.

8.1 Conclusions

648. Several important conclusions can be derived from this detailed review paper:

1. Research published in the past 5 years has clarified important issues germane to thyroid toxicology, and suggests endpoints and assays that should be considered for research and development and, if possible, current or future use in assay protocols (in addition to those initially recommended).

2. Many of the current *in vivo* screens and tests were originally designed to evaluate toxicant effects on reproduction and development. These protocols can be modified to test for thyroid toxicants by the addition of specific endpoints acquired at specific developmental time points. Although selected EPA and/or OECD protocols are adequate in their dosing regimen and timing of treatment, they will require adaptation in the future for the timing of any newly developed thyroid endpoints designed to effectively evaluate toxicant effects on thyroid hormone action.

3. Thyroid hormones and thyroid histology are essential endpoints reflective of thyroid toxicity; in fact, all known thyroid toxicants have been identified by their ability to influence these endpoints. However, toxicants acting at different sites within the HPT axis appear to produce a different profile of hormone changes in relation to thyroid weight and histology. In addition, toxicant effects on the HPT axis may change over duration of treatment; thus, repeated sampling is important to capture dynamic events that may be informative.

4. Endpoints of thyroid hormone action, both in development and in the adult, could greatly enhance the power of a data set generated on a specific thyroid toxicant to inform regulatory bodies. However, endpoints of thyroid hormone action are not well characterized within the context of toxicological research (i.e., dose sensitivity and specificity). Thus, these will require additional study before incorporated into existing screens and tests.

5. Thyroid endocrinology and biochemistry are remarkably conserved across vertebrate taxa (as discussed in the beginning of this section).

6. A significant number of new reagents have become available, including identified genes and antisera, which will better support homologous assay development in non-mammalian vertebrates.

8.2 Overall Strategy for Thyroid Screening and Testing

649. Thyroid assays using nonmammalian vertebrates can provide important information about potential thyroid toxicity in wildlife species. These assays may also have generalizable applicability to vertebrates considering the degree to which the thyroid system is conserved across taxa. Capturing endpoints of thyroid toxicity in pre-existing *in vivo* rodent assays designed to evaluate reproductive and developmental toxicities provides the advantage of adding value to these assays without the use of additional animals. Moreover, careful design of the timing of toxicant exposure and thyroid endpoint acquisition in these assays can provide important information about the ability of specific toxicants to exert effects on development or on the adult by disrupting the thyroid system. This overall design feature has several implications as users of this document consider development of standardized approaches to evaluate thyroid toxicity and for the potential health consequences of these effects. If a tiered approach to thyroid toxicity testing is being considered, then the first line of screening should include measures of thyroid function, which represent the hallmark features of antithyroid actions of all known thyroid toxicants (Brucker-Davis 1998). These measures include, in general, circulating levels of thyroid hormone and measures of thyroid histology. Thus, these endpoints should be incorporated into rodent assays designed to be part of an initial tier (e.g., acute studies). Chronic studies important to evaluate potential carcinogencity may have more apical endpoints (e.g., endpoints of thyroid hormone action) for added information. The second implication is that thyroid endpoints must be integrated into protocols in a manner that minimizes false-negatives. Thus, the following points should be considered when incorporating thyroid endpoints into existing experimental protocols.

A. *Development of the HPT Axis*

650. The HPT axis develops with a time-course specific for the animal (across taxa). For example, metamorphosis in fish and amphibians represents a time when many changes are occurring, including changes in the sensitivity to thyroid hormone or in other hormones involved in regulation of the system. In rodents, the negative feedback action of thyroid hormone on the hypothalamus and pituitary does not fully develop until the first week of life in the rat. Fukiishi and Hasegawa (1985) reported that rat fetal serum TSH concentration declined significantly between 20 and 21 days of gestation, reaching a low level at delivery, and remained low for several days after birth. T_3 suppressed serum TSH concentration further in a dose-responsive manner when given to fetuses on day 20 of gestation at 0.13 to 2.0 micrograms/100 g body weight of the estimated body weight. The responses of serum TSH levels and thyroid weights to PTU treatments differed with gestational age. Thus, they concluded that negative feedback control by T_3 of serum TSH concentration exists in rat fetuses as early as day 20 of gestation, but it differs from that found in adult rats. In addition, Taylor *et al.* (Taylor et al. 1990a) found that thyroidectomy did not cause an increase in TRH mRNA levels of the hypophysiotropic PVN until PND 7, indicating that the hypothalamic limb of the negative feedback system developed later than that of the pituitary limb. Moreover, Nikrodhanond et al. (2006) have provided compelling evidence that the hypothalamus plays the dominant role (compared to negative feedback by TH) in regulating serum TH levels. Therefore, while thyroid endpoints of serum hormone levels and thyroid histology should be taken during the first postnatal week (e.g., PND 5 in OECD draft guideline 426) the interpretation of toxicant effects on these endpoints should take into consideration the development of the HPT axis.

B. *Duration of Treatment*

651. There are two competing issues when considering the duration of toxicant treatment and thyroid endpoints in vertebrates. The first is that because of the storage capacity of the thyroid gland, it may require several days before toxicant effects are observed on circulating levels of thyroid hormones and/or thyroid histology. In contrast, because of the potential compensatory mechanisms of the HPT axis and other tissue-level compensatory responses, thyroid toxicants may have a rapid effect on serum hormone

levels that are "compensated" after some time. Moreover, it is clear that toxicants acting on the HPT axis through a different mechanism may elicit different compensatory responses and may require different durations. Considering this complexity of the HPT axis, thyroid endpoints should be captured at multiple time-points. In adult rats, this may be represented by an early (48 hours) and late (14-28 days) time point. In development, this would be represented by two or three times during early postnatal development (e.g., P5, P15, and P21) as well as an adult time point.

C. *Endpoints of Thyroid Function and Thyroid Hormone Action*

652. Measures of thyroid function include serum hormone levels and thyroid histology. These endpoints represent the foundation of any assay for anti-thyroid activity. Changes in these measures of thyroid function associated with toxicant exposure represent the sole source of information by which thyroid toxicants have been identified, and hundreds of chemicals have been identified this way (see review by Bruker-Davis, 1998). There are a number of potential endpoints of thyroid hormone action, both in the adult and in the developing animal. These endpoints are extensively discussed in Chapters 3 and 4 and could be utilized in experimental protocols at this time. However, more research and development would be needed before they are considered for validation in programs where needed.

8.3 Endpoints of thyroid hormone action that represent potentially useful endpoints.

653. The following overview includes endpoints and assays considered to be a priority for research and development as well as those available for validation, so that regulatory programs may further develop and/or incorporate those that will be most valuable for their particular purposes. For a specific list of existing or potential future assays see Table 8-1. *In vitro* screens are described as potential ways to identify thyroid toxicants that act by very specific mechanisms (e.g., binding to TRs), that could be adapted to a high throughput platform. However, because of the complexity of regulation of the thyroid system, a very large and potentially unwieldy number of *in vitro* screens would have to be developed and employed to provide a comprehensive evaluation of all known mechanisms of thyroid toxicity. Moreover, because of the many points of regulation of thyroid endocrinology that can respond to fluctuations in the thyroid system, even these isolated *in vitro* screens would not be as comprehensive as an *in vivo* screen or test.

a. *In Vitro* screening assays

Research and Development:

654. A number of *in vitro* screening assays are described in this DRP. Generally, these fall into two categories—*in vitro* systems that 1) specifically examine receptor binding and activation, and 2) allow observation of the consequences of disrupting specific modes of action. The following *in vitro* assays are in different states of research and development. None of them have been validated for use as screening assays, and all of them need various amounts of development before they could enter into validation.

655. *In vitro* thyroid hormone receptor (TR) binding and activation assays are equivalent to estrogen and androgen receptor binding and activation assays. They can be made to accommodate high throughput and can identify thyroid toxicants that interact directly with thyroid hormone receptors. All vertebrates have TRs; their comparative structure and the kinetics of T_3 binding to these TRs are quite similar. Therefore, it is theoretically possible that xenobiotics will bind to all vertebrate TRs with the same characteristics. This needs to be tested before being assumed.

656. *In vitro* assays that allow examination of thyroid hormone action may be useful, but certain disadvantages exist. For example, GH_3 cells may be used to detect generalized disruption of TR action in a manner analogous to the ESCREEN for estrogenic/antiestrogenic chemicals. Although this assay may be

prone to false positives, it could be used as a tool to prioritize chemicals in conjunction with binding assays because these cells have both TRα and TRβ receptors and they respond to T_3 with proliferation.

657. Other *in vitro* assays allow the investigator to evaluate the effects of chemicals on specific modes of actions. Most of these assays use cell lines that can address specific modes of action of thyroid disruption. For example, FRTL-5 cells can be used for their ability to concentrate iodide. Purified thyroperoxidase or a crude extract can be used to test for the ability of chemicals to block this enzyme.

658. The *in vitro* assays are most useful in exploring specific modes of action, but it would be unrealistic to incorporate *in vitro* tests that cover all possible points of thyroid disruption across taxa into a screening and testing battery—a large battery of *in vitro* tests would have to be assembled to allow chemicals to be tested for all aspects of thyroid toxicity. Thus, it would appear to be most effective to focus on adapting existing *in vivo* assays for thyroid endpoints. As these would be added endpoints to existing assays, little or no increase in animal usage would be required.

Possible Inclusion in Validation at This Time:

659. No *in vitro* assays are currently ready to validate in an existing screening battery. Several of the *in vitro* assays discussed in this document could be considered for validation after a limited amount of research and development.

b. *In Vivo* screening assays

Research and Development:

660. It is important to recognize that all known thyroid toxicants (among hundreds) have been identified using endpoints of serum hormone levels and thyroid histology as endpoints of toxicity. Therefore, it is essential that these endpoints continue to be employed – and improved – to serve as bellwethers of thyroid toxicity. However, it is equally important to recognize that the pattern of changes in these endpoints of serum thyroid hormones and thyroid histology are not always consistent with an idealized model of thyroid endocrinology as revealed by studies using PTU or MMI (Zoeller 2005). Appendix A presents an analysis of three thyroid toxicants, and Appendix B presents a statistical comparison of thyroid activity measured across data sets. In these cases, and even perhaps in cases where the profile of endocrine changes are fully consistent with this idealized model, it may be of value to obtain measures of thyroid hormone action as further information on the adverse effects of the observed changes in circulating levels of thyroid hormone. Thus, the assays below are discussed as potential sites of thyroid hormone action that could be developed for such tier-2 studies.

661. In general, the *in vivo* screening assays are relatively short-term treatments of toxicants during peripubertal or adult life stages (e.g., OECD 407 and the male and female pubertal assays). In the future, simpler, less costly, and more informative endpoints may be developed to replace labor-intensive and expensive endpoints. For example, measuring thyroid gland T_4 content, as proposed by McNabb et al. (2004a, 2004b), may be a more sensitive indicator of TSH stimulation than current endpoints in the face of specific toxicants. Endpoints such as body weight or behavioral activities are affected by severe thyroid hormone insufficiency, but are not likely to be sensitive to small changes in circulating levels of thyroid hormones. There are few other *in vivo* endpoints of thyroid hormone action in adults that are well developed, and research in this area is needed.

Possible Inclusion in Validation at This Time:

662. These assays can provide important information about thyroid toxicants if strategic endpoints are included as described in this document. As described earlier in this chapter, endpoints more relevant to thyroid hormone changes at different life stages, or to changes that occur following exposure to chemicals that alter thyroid hormone levels, could be added to existing *in vivo* assays with little alteration to the number of animals utilized.

c. In Vivo Tests

Research and Development:

663. The *in vivo* tests include a number of developmental tests such as the OECD prenatal toxicity test or the one- or two-generation reproductive toxicity test. These tests can be modified to include measures of development that may be sensitive biomarkers of thyroid disruption. These future endpoints will likely be measures of histogenesis. There are a number of endpoints associated with neuronal differentiation and migration in the cerebellum and cerebral cortex (during cerebral cortical layering) in the developing brain. These endpoints may be highly sensitive to thyroid hormone insufficiency and would clearly reflect adverse effects. Endpoints for brain development are still progressing and are not yet ready for validation in any regulatory testing program.

Possible Inclusion in Validation at This Time:

664. As described earlier (chapter 4 and chapter 8), additional time points for thyroid hormone measurement could accompany existing tests (such as the two-generation reproduction assay) so that developmental changes in thyroid hormone would be more accurately monitored.

d. Methods to integrate results from multiple species (including Table 8-2 below, showing points of disruption across taxa).

665. Interpreting results from several vertebrate taxa will provide useful information on cross-taxa similarities and differences. Two key considerations for interpretation of data are: 1) different classes of vertebrates, and genera/species within those classes, likely have specific metabolic capacities or other physiological mechanisms that may render them particularly sensitive or insensitive to any one thyroid toxicant; and 2) it is likely that specific chemicals that interfere directly with thyroid hormone ssynthesis, transport, or signaling will exert these effects across vertebrate taxa; however, the specific effects of thyroid hormone (and disruption) in different taxa will vary considerably. We are just beginning to investigate these issues and we cannot expect to be able to derive broad inferences at this time.

8.4 Implications

666. The goal of this document is to provide a detailed review of the current literature of thyroid endocrinology and a basis for the strategic design of screens and tests to effectively identify environmental thyroid toxicants across taxa. The endocrine system is complex, and there are large gaps in our understanding of this system and the role it plays in development and physiology. Moreover, a reasonably comprehensive review of a variety of endpoints has been provided so that a broad perspective of available endpoints could be realized. The complexity of the endocrine system combined with large data gaps and endpoints uncharacterized in toxicological studies undoubtedly calls for ongoing research and development, as well as frequent re-evaluation and upgrading of the thyroid endpoints and assays used for regulatory purposes.

667. Table 8-1 shows existing or potential assays across all four taxa of interest, including a brief discussion of the strengths and weaknesses of each endpoint. Although reasonably comprehensive, the text provides a more complete discussion of the issues underlying these assays. Table 8-2 shows the primary targets of disruption across all four taxa with a brief discussion of their significance. Finally, Table 8-3 provides a listing of endpoints of thyroid hormone action that may be the most likely to be incorporated into various assays in the future based on current research. These endpoints represent the best professional judgement of the various chapter authors.

Table 8-1 Existing or Potential *In vivo* and *In vitro* Assays

Assay Name	Species	Primary Thyroid-Related Endpoints	Target Effects Relevant to the Thyroid System	Strengths	Weaknesses	Additional Endpoints to Consider for Improvement
SCREENING ASSAYS UNDER DEVELOPMENT/VALIDATION						
OECD TG 407 Repeated dose 28-day oral toxicity study	Rat	Total serum T_4, TSH, Thyroid weight and thyroid histology.	Changes in circulating levels of TH; hypertrophy or proliferation of thyroid follicles.	Straightforward add-on; circulating levels of TH can be related to human thyroid function; follicular proliferation reflects TSH increase; thyroid histology not particularly sensitive to confounders , tumor occurrence important cancer endpoint.	Time course data lacking for compensatory changes; response to stress not characterized	Possible cardiovascular function (heart rate, blood pressure); possible body temperature. Possible liver endpoints (Malic enzyme).
OECD TG 414 Prenatal Development Toxicity Study	Rat	Total serum T_4, TSH, thyroid weight and thyroid histology.	Changes in circulating levels of TH hypertrophy or hyperplasia of thyroid follicles; possible reproductive development (e.g. testes)	Straightforward add-on; circulating levels of TH can be related to human thyroid function; follicular proliferation reflects TSH increase; thyroid histology not particularly sensitive to	Prenatal exposure is less well studied for thyroid toxicants and for TH insufficiency. Serum volume is low, which requires pooling for assays. This reduces power. Few additional endpoints of TH action in the fetus.	

Assay Name	Species	Primary Thyroid-Related Endpoints	Target Effects Relevant to the Thyroid System	Strengths	Weaknesses	Additional Endpoints to Consider for Improvement
				confounders , tumor occurrence important cancer endpoint; time-course data can be collected.		
OECD 415/416 1- and 2-Gen Reproductive Toxicity Studies	Rat	Total serum T_4, TSH, thyroid weight and thyroid histology.	Changes in circulating levels of TH hypertrophy or hyperplasia of thyroid follicles; possible reproductive development (e.g. testes)	Straightforward add-on; circulating levels of TH can be related to human thyroid function; follicular proliferation reflects TSH increase; thyroid histology not particularly sensitive to confounders , tumor occurrence important cancer endpoint; time-course data can be collected.	This is a very large study design that could capture elements of the consequences of TH disruption.	Many developmental events that are influenced by thyroid hormone could be added on. These include myelination, cortical lamination, cerebellar development. Also, possible cardiovascular development.
OECD 421/422 Reproductive/ Developmental Toxicity Study	Rat	Total serum T_4, TSH, thyroid weight and thyroid histology.	Changes in circulating levels of TH hypertrophy or hyperplasia of thyroid follicles; possible reproductive development (e.g. testes)	Straightforward add-on; circulating levels of TH can be related to human thyroid function; follicular proliferation reflects TSH increase; thyroid	This is a very large study design that could capture elements of the consequences of TH disruption.	Many developmental events that are influenced by thyroid hormone could be added on. These include myelination, cortical lamination, cerebellar development. Also, possible

Assay Name	Species	Primary Thyroid-Related Endpoints	Target Effects Relevant to the Thyroid System	Strengths	Weaknesses	Additional Endpoints to Consider for Improvement
				histology not particularly sensitive to confounders , tumor occurrence important cancer endpoint; time-course data can be collected.		cardiovascular development.
OECD 426 Developmental Neurotoxicity Study		Total serum T_4, TSH, thyroid weight and thyroid histology.	Changes in circulating levels of TH hypertrophy or hyperplasia of thyroid follicles; possible reproductive development (e.g. testes)	Straightforward add-on; circulating levels of TH can be related to human thyroid function; follicular proliferation reflects TSH increase; thyroid histology not particularly sensitive to confounders , tumor occurrence important cancer endpoint; time-course data can be collected.	This is a very large study design that could capture elements of the consequences of TH disruption.	Many developmental events that are influenced by thyroid hormone could be added on. These include myelination, cortical lamination, cerebellar development. Also, possible cardiovascular development.
Amphibian Metamorphosis Assay (21-Day Assay with initiation at NF stage 51)	*Xenopus laevis*	Hind limb length; thyroid gland histology; whole body length; developmental stage; mortality.	Normal, delayed, or accelerated metamorphosis from tadpole to frog.	May be more sensitive than tail resorption alone; more comprehensive than other Tier I screens for thyroid;	Toxicant metabolism is unknown across taxa.	T_4 levels

Assay Name	Species	Primary Thyroid-Related Endpoints	Target Effects Relevant to the Thyroid System	Strengths	Weaknesses	Additional Endpoints to Consider for Improvement
				relatively short; can accommodate other biochemical and molecular biomarkers.		
POTENTIAL *IN VITRO* SCREENING ASSAYS						
In vitro receptor binding	Isolated recombin-ant receptors from any vertebrat e	T_3 binding to receptor	May be important mechanism by which some toxicants could interfere with thyroid signaling	Solid state binding assays available; low rate of false positive; appropriate for high through-put	Receptor binding not fully characterized as a mechanism; high false negative; no metabolic activation; solubility	
Receptor activation using recombinant receptors (from any vertebrate)	Various types of cell lines	Functional assay to define pharmacology	Tissue end organ effects of T_3	Can determine agonist or antagonist properties; system can be manipulated, optimized, etc.; readily adapted to high through-put	limited metabolic activity; cell wall (yeast)	
Thyroid Peroxidase (TPO) using lactoperoxidase		Iodine organification	Iodine organification	Sensitive; unlikely to produce false positives; *In vitro* uses fewer animals; could be adapted to high through-put application	No rodent or human TPO available; high false negative due to specificity; only one of many MOAs that affect hormone levels.	
Binding to serum proteins (TTR, TBG)	Rat, human, others by design	Displacement of T_4 from proteins; potentially reduce serum T_4;	May be a mechanism by which some chemicals cause serum T_4 reduction; potentially may reduce T_4 uptake into tissue	Well-characterized; can be modified for high through-put; may be predictive of chemicals that alter fetal T_4	Many other MOAs affect serum hormones in addition to this; TTR knock-outs do not support relevance to adverse effects.	

Assay Name	Species	Primary Thyroid-Related Endpoints	Target Effects Relevant to the Thyroid System	Strengths	Weaknesses	Additional Endpoints to Consider for Improvement
			including brain.			
Deiodinase	Frog, fish, possibly mammal	Conversion of T_4 to T_3 (outer ring deiodinase) or reverse T_3 (inner ring deiodination)	Potentially a mechanism by which tissues regulate their sensitivity to thyroid hormone	Well characterized assay; important endpoint for tailored tests	Not a single assay (three types); tissue and species differences in deiodinases	
Glucuronidation	Rat, others as available	T_4 glucuronidate	T_4 deactivation, reduction of circulating levels	Well-characterized; *in vivo* exposure, *ex vivo* assay; inducible; not as sensitive to diurnal rhythm or stress	Very specific; high false negative; somewhat laborious	
GH_3 cell assay	Rat	Growth/proliferation; normal morphology of cell signals; can be constructed to identify agonist/antagonist	Local tissue effect of T_3	High through-put adaptability; uses fewer animals; can detect agonist or antagonist activity	Specific for TR binding; high false negative	
CURRENT TESTS (WITH THE OPTION TO ADD THYROID ENDPOINTS)						
Mammalian one- or two-generation	Rat/ mouse	T_4/TSH levels, thyroid weight and histopathology being considered as add-on	Hormone levels and histopathology would provide potential measure of thyroid dysfunction during development	Would provide at least some thyroid specific endpoints; provides a postnatal developmental hormone profile; doesn't use additional animals	Does not provide endpoints of specific hormone effects in tissue	(PND4, PND21, Adult) In addition to hormone levels and thyroid histopathology: Serum binding proteins; serum Tg; Thyroid gland hormone content; Cortical lamina (BrdU in utero); cerebellar histology (P5-15); granule cell apoptosis (P5-10); Oligo # or anterior commissure area; heart

Assay Name	Species	Primary Thyroid-Related Endpoints	Target Effects Relevant to the Thyroid System	Strengths	Weaknesses	Additional Endpoints to Consider for Improvement
						development.
TESTS CURRENTLY BEING DEVELOPED						
Fish two gen	Fat head minnow, medaka, zebrafish, sheeps-head minnow	T_4 levels (whole body/serum/tissue), thyroid weight and histopathology	Thyroid status	Non mammalian test; thyroid function effects over time/development stages	May be insensitive to thyroid toxicants; tissue measures may be inaccurate or laborious; few TSH methods (may require development); T_3 not currently included	TSH, T_3 measurements; deiodinase assay; gill chloride
Avian Two-Generation Assay	Japanese quail	Circulating T_4, T_3, TSH, thyroid weight, thyroid histology, bone length, skeletal endpoints; thyroid gland hormone content; body weight/growth rate	Developmental profile of thyroid function, assay of thyroid hormone-sensitive tissues (skeleton); HPT axis activation	Doesn't require sacrifice; relatively inexpensive, simple, quick; easily validated; new information indicates gland TH content is sensitive and reliable.	T_4 and T_3 are highly variable; no TSH assays; histopath is labor intensive. Body weight very insensitive	
Amphibian Growth and Reproduction Test	*X. (Silurana)* tropicalis	Hindlimb length, thyroid gland histology, whole body length, developmental stage (rate)	Normal, delayed or accelerated development from tadpole to frog	May be more sensitive than tail resorption alone; more comprehensive than other Tier I screens for thyroid; relatively short; can accommodate other biochemical and molecular biomarkers. The battery may also provide advanced developmental, sexual differentiation and other	Toxicant metabolism is unknown across taxa.	TSH, T_4, and T_3, steroid hormones, and aromatase

Assay Name	Species	Primary Thyroid-Related Endpoints	Target Effects Relevant to the Thyroid System	Strengths	Weaknesses	Additional Endpoints to Consider for Improvement
				reproductive endpoints in addition to the previously mentioned thyroid-related endpoints (i.e., the test is all-inclusive).		
CONSIDERATION FOR RESEARCH AND DEVELOPMENT						
Avian embryo assay	Japanese quail	Toxicant application to external air cell membrane; thyroid endpoints during embryonic development and 1-day chick including gland hormone measurements; histopathology; skeletal x-ray	Developmental endpoints of thyroid function and thyroid hormone action	Developmental times may be more sensitive to thyroid-specific toxicants	Unknown sensitivity to thyroid hormone or thyroid toxicants	
Larval fish assay	Larval fish	Transition from larval to juvenile form; potential large number of morphological changes associated with transformation (e.g., gut, fins, mouth) Development/growth, hormone content, histopathology	Normal, delayed, or accelerated morphogenesis from larval to adult form	Developing larval fish have largely been ignored in thyroid studies but may prove to be highly susceptible to thyroid disruption	Techniques will need to be refined for thyroid analyses of extremely small fish. Relevance to other taxa, especially mammals, is unknown. This assay requires further development and refinement, standardization and validation	
Flounder metamorphosis assay	Flounder	Transition from planktonic to benthic; potential large number of morphological	Normal, delayed, or accelerated morphogenesis from larva to juvenile	Straightforward morphological and behavioral endpoints, reflecting integrated	Does not consider other components of the fish thyroid cascade, such as	

Assay Name	Species	Primary Thyroid-Related Endpoints	Target Effects Relevant to the Thyroid System	Strengths	Weaknesses	Additional Endpoints to Consider for Improvement
		changes associated with metamorphosis (e.g., eye migration, pigment asymmetry, stomach formation)		effects of thyroid hormones	central T_4 production (Brain-pituitary-thyroid axis). Relevance to other taxa, especially mammals, is unknown. This assay requires further development and refinement, standardization and validation	

Table 8-2 Points of Disruption across Taxa

Primary Target	Mammals (Chapter 3)	Birds (Chapter 7)	Amphibian (Chapter 6)	Fish (Chapter 5)
Thyroid				
Iodide Uptake	Thyroid cells in all vertebrates, and cells of the endostyle in some invertebrates, concentrates iodide by the action of (at least) the sodium-iodide symporter (NIS). This protein is homologous in all vertebrates, but the comparative aspects of this protein in different vertebrates has not been well characterized. Therefore, while NIS inhibition is a potentially important point at which thyroid disruption could occur, research may show that specific chemicals (e.g., perchlorate) may be more potent in some vertebrates than in others. The protein Pendrin also may be an important target of toxicant actions. Pendrin in mammals is associated with iodine transport through the apical membrane into the region where peroxidase activity organifies the iodine. Likewise, Pendrin is expressed in all vertebrates and plays different roles in thyroid physiology.			
Iodine Organification	The thyroperoxidase enzyme, similar to NIS, may be different enough among the taxa that it will respond differently to specific EDCs. Further research is required to clarify this issue. Moreover, TPO requires the activity of several enzymes to generate hydrogen peroxide in a location-specific manner. The DUOX proteins accomplish this in association with addition proteins that have not been well characterized. The comparative aspects of these proteins have not been well studied, and may be important in toxicological research.			
Thyroglobulin Degradation	The initial solubilization of Tg occurs in the colloid by the combined actions of Cathepsins B, L, and D. Further degradation occurs in secondary lysosomes as Tg is taken up by endocytosis and further degraded by Cathepsins. Some exogenous factors are known to inhibit these enzymes – specifically Lithium. However, the comparative aspects of these steps, which are present in all vertebrates, are poorly studied.			
Effects	The first effect of direct inhibition of thyroid function will be the reduction in thyroid hormone synthesis and secretion. There are a great many variables that differ among vertebrate taxa that will influence this effect. Specifically, differences among vertebrates in serum half-life for thyroid hormones, the storage capacity of the thyroid gland for thyroid hormone, and the relative sensitivity of thyroid hormone synthesis to EDCs acting on the NIS or TPO, will be important to consider when comparing the relative potency of EDCs among taxa.			
Hormone Assays	In general, hormones are measured by radioimmunoassay (RIA). This is especially true for serum measurements and can also be true for tissue measurements, though additional physical measurements are also performed (e.g., HPLC, MS/GC). It is essential when considering the specific RIA, especially if this is a commercial kit, that the assay is validated. For thyroxine (T_4) and triiodothyronine (T_3), the chemistry of the hormone is identical among all vertebrates, but the specific matrix (e.g., serum) will be different. Therefore, it is possible – even common – that an RIA kit developed for humans will not be valid for use to measure the same hormone in the serum of other animals such as rodents, frogs, etc. Assay validation is accomplished by demonstrating that a dilution of matrix (e.g., serum) produces a linear function that is parallel to the standard curve. In addition, the addition of known quantities of hormone (e.g., T_4) to matrix (e.g., serum) should produce the expected results. Protein hormones such as TSH are most often not valid in heterologous assays in which the antibody is generated to a TSH from one species for use in another species. Thus, one should not predict that the rat TSH RIA will be valid for mouse. However, following the above method for validation will demonstrate empirically whether the assay is valid or not. Finally, the standard curve should never be used between zero and the lowest standard (i.e., extrapolation). Rather, only interpolating between standards on a valid curve is appropriate. Thus, the specific RIA must be valid (i.e., parallel), but the standards must also be calibrated such that control animals are on the middle of the standard curve.			

Primary Target	Mammals (Chapter 3)	Birds (Chapter 7)	Amphibian (Chapter 6)	Fish (Chapter 5)
Specific Assays	RIA kits are commercially available for T_4, T_3, free T_4, free T_3, and TSH. The T_4 kit most commonly used (a human serum-based kit) is not well calibrated for T_4 in rats. Measurement of fT_4/fT_3 are vulnerable to changes in binding proteins and may be invalid. Volumes of serum required for the RIA can be large and therefore difficult to obtain in small animals (pups).	RIAs and ELISAs are in common use for thyroid hormones. Although T_4 and T_3 are chemically identical to thyroid hormones in all vertebrates, including humans, serum components may differ among taxa/species such that human kits are not valid. Validation procedures should be instituted. No immunoassay exists for avian TSH, but could be developed. Serum volumes required for multiple assays often limiting.	RIAs and ELISAs are in common use for thyroid hormones. Although T_4 and T_3 are chemically identical to thyroid hormones in all vertebrates, including humans, serum components may differ among taxa/species such that human kits are not valid. Validation procedures should be instituted. No immunoassay exists for amphibian TSH, but could be developed. Serum volumes required for multiple assays often limiting. Volumes available for analysis may be low and "whole body" measures may be required.	RIAs and ELISAs are in common use for thyroid hormones. Although T_4 and T_3 are chemically identical to thyroid hormones in all vertebrates, including humans, serum components may differ among taxa/species such that human kits are not valid. Validation procedures should be instituted. No immunoassay exists for fish TSH, but this could be developed. Serum volumes required for multiple assays often limiting. Volumes available for analysis may be low and "whole body" measures may be required.
	Note: TSH is present as a protein dimmer in the pituitary of all vertebrate taxa. However, this large glycoprotein is different enough among taxa – and even between species within a class – that assays must be tailored for the specific TSH or a closely related one.			
Thyroid Measures	Thyroid gland weight Histopathology -May represent an integrated measure of thyroid function over time. Signs of hyperplasia may indicate susceptibility to cancer; however this is controversial. -Measure of stored T_4/T_3 not routinely performed but may be important.	Thyroid gland weight Histopathology -Both require training. Histopathology not validated for avian EDC research.	Thyroid structure differs from mammals and among amphibian species. Histopathology has not been validated for endocrine or EDC studies.	Thyroid structure differs from mammals and among fish species. Histopathology has not been validated for endocrine or EDC studies.
Adverse Effects	Not routinely measured. Could include a variety of developmental and physiological endpoints. These are reviewed in Chapter 4. Developmental endpoints may be most sensitive. Potential assays are reviewed in Chapter 4.	Not routinely measured. Could include a variety of developmental and physiological endpoints. Developmental endpoints may be most sensitive. Potential assays are reviewed in chapter 7.	Amphibian metamorphosis being actively investigated as potential measure of EDC adverse effects on development. Many reagents/methods approaching validation.	Not routinely measured. Could include a variety of developmental and physiological endpoints. Flounder metamorphosis may be a simple and quantitative assay for EDC adverse effects through multiple modes of action.
Hormone Metabolism				
Serum Binding Protein displacement	YES	YES	YES	YES
	Both TTR and Thyroxine binding globulin (TBG) are present in mammals; TTR is present in all vertebrates. TTR does not appear to be			

Primary Target	Mammals (Chapter 3)	Birds (Chapter 7)	Amphibian (Chapter 6)	Fish (Chapter 5)
	developmentally regulated, or regulated by thyroid status, in mammals; however, TBG is very sensitive to thyroid status in adult rodents. The sensitivity of TBG expression in developing mammals is poorly understood, but may be important in toxicological studies. The role of T_4/T_3 displacement in the etiology of adverse effects induced by thyroid toxicants is suspected but not clear.			
Effects	A prevailing theory is that if T_4 (and/or T_3) is displaced from serum binding proteins, then the hormones will be more rapidly removed and adverse effects of thyroid hormone insufficiency will result. However, humans with defective or absent binding proteins have altered TH levels, but no symptoms of hypothyroidism. Moreover, TTR knock-out mice have low serum hormone levels, but normal tissue levels (including brain). However, this mode of action may contribute to effects of EDCs on thyroid hormone levels. The three major thyroid hormone binding proteins – transthyretin, thyroxine binding globulin, and albumin – are expressed in different ratios in different vertebrates and differ somewhat in their structure.			
Conjugation and Glucuronidation	Glucuronidation and conjucation (sulfation and sulfonation) occurs in all vertebrates and represents important pathways of T_4 degradation both in the liver and in target tissues. The enzymes required for accomplishing these steps have not been widely studied for their toxicological relevance. It is highly likely that induction of these enzymes will reduce serum thyroid hormones, but there is little evidence that xenobiotic-induced redtion of the expression or activity of these enzymes will have effects.			
UDPGT induction	YES	YES	YES	YES
Effects	Current theory is that induction of these enzymes by EDCs can increase their clearance (decreasing serum half-life) and causing adverse consequences mediated by thyroid hormone insufficiency. Evidence supports this concept, but there are UDPGTs selectively directed against T_4 or T_3 and EDCs may differ in their ability to induce one or both of these.			
Tissue Uptake				
T_4 transporters	YES	YES	YES	YES
T_3 transporters	YES	YES	YES	YES
Effect	Several recent papers strongly suggest that T_3-transporters are expressed selectively on nerve cells within the central nervous system and that defects in this protein (MCT8) causes mental retardation and neurological deficits. Few endocrine or EDC studies have been performed, but these may be important.	Little information is available in birds for the existence of cellular transporters for T_3 and T_4. May be important site of EDC action.	There is some evidence that cells such as red blood cells have active TH transport in amphibians. Little work has been performed to identify these transporters and to characterize their importance in thyroid hormone signaling or as targets of EDC action.	More evidence exists for active transport mechanisms for cellular uptake in fish, but little evidence for the role of these proteins in physiology or effects of EDC on their function.
TRs				
α/β Isoforms	YES	YES	YES	YES
Effects	It is becoming clear that the different TR isoforms mediate different actions of thyroid hormone on development and physiologic of all vertebrates. There is more information available in mammals, but enough information exists in some representatives of other taxa to make this conclusion. This is important because there may be EDCs that selective affect specific TR isoforms. Although this has not been identified for any EDC, it would complicate the identification of adverse effects because assays would have to be designed to identify TR isoform-specific endpoints. A second important issue is that while T_3 binds to all TRs, we do not know if individual EDCs bind to all TRs equally. This is not likely. Therefore, TR binding as an EDC screen may require TRs from different taxa to address this issue. Finally, the actions of TRs in different vertebrates are different. In addition, these actions differ at different life stages. Therefore, endpoints of EDC effects on TR actions must be strategically designed.			

Primary Target	Mammals (Chapter 3)	Birds (Chapter 7)	Amphibian (Chapter 6)	Fish (Chapter 5)
Deiodinases –				
	There are two or three deiodinase enzymes in each taxa. These proteins share a great deal of similarity, but no studies have evaluated the effects of EDCs across the various deiodinases. However, considering that tissue expression of deiodinases controls sensitivity of the tissue to thyroid hormone, this may be an important point at which EDCs could disrupt thyroid hormone signalling .			
HPT axis	In all vertebrates, the dynamic relationships between the hypothalamus, pituitary and thyroid are functionally similar. Differences among vertebrates exist in some of the hypothalamic peptides controlling pituitary-thyroid function, but the HPT axis is functionally similar in all vertebrates.			

Table 8-3 Well-Established Endpoints of Thyroid Hormone Action that may be Recruited for Toxicity Studies

Specific End Points	Mammals (Chapter 3)	Fish (Chapter 5)	Amphibians (Chapter 6)	Birds (Chapter 7)
Brain			**CNS Restructuring** - Restructuring of medulla and cerebellar neurons, genetically programmed regression/disappearance of giant neurons, Mauthner cells and Rohon-Beard neurons (Hughes, 1957; Moulton et al., 1968). Purkinje cells, lateral motor column neurons, and the dorsal root ganglia neurons further differentiate during metamorphosis (Hoskins, 1990)	
Genes	RC3/Neurogranin	Few studies have focused on the effects of thyroid hormone on the fish brain. Thus, endpoints of TH action in fish brain are not available at this time.	**Corticotropin Releasing Factor (CRF)** - evidence supports a role for CRF in the regulation of TSH during metamorphosis (Denver et al., 2002; Okada et al., 2003).	Few studies have focused on the effects of thyroid hormone on the bird brain. Thus, endpoints of TH action in bird brain are not available at this time.
	Thyrotropin-releasing hormone		**Thyroid Stimulating Hormone (TSH)** - TSH genes have been cloned (cDNAs) in *X. laevis* (Buckbinder and Brown, 1993) encoding for both subunits and used as a diagnostic tool to measure the time course of expression through metamorphosis	

Specific End Points	Mammals (Chapter 3)	Fish (Chapter 5)	Amphibians (Chapter 6)	Birds (Chapter 7)
	Purkinje cell-specific protein-2		**Thyroid Hormone (TH) and Thyroglobulin –** biochemical measurements of glandular and plasma levels	
	Reelin		**Other Potential Genes regulated by TH** – Tail 1/3 – zinc finger (BTEB), Xh20 (protein disulfide isomerase)	
	Hairless			
	Recent microarray studies reveal a large number of genes that are regulated by thyroid hormone, but many of these have not been pursued with focused hybridization studies.	It is important to recognize that there may be significant and important differences in the role of thyroid hormone in development and physiology among species within a single class of vertebrates. Therefore, as one considers developing endpoints of TH action in species that have not been used as model systems, it is possible that important differences among species will become apparent, rendering these efforts difficult to predict at the outset.		
		Flounder settling behavior (indicates effect on brain)		Type II deiodinase (not well established)
Developmental Events	Cortical neuronal migration and establishment of cortical layers.	Flounder metamorphosis specific endpoints (e.g., eye migration) may be important.	**Thyroid gland** – development and histology during metamorphosis	Bone Maturation
	Cerebellar development. Developmental timing of granule cell proliferation, migration across the mitral layer and survival/apoptosis in the internal granule layer.		**Limbs -** Hind limb differentiation and forelimb development and emergence	
	Cerebellar Purkinje cell arborisation		**Other Metamorphic Restructuring/Resorption and Biochemical Changes** – neuorns, intestines, gills, lungs, tail. Biochemical changes also occur.	
	Myelination. Thyroid hormone plays a specific role in differentiation of oligodendrocytes and astrocytes from a common progenitor.		**General rate of development -** Measured by development stage and hind limb length	

Specific End Points	Mammals (Chapter 3)	Fish (Chapter 5)	Amphibians (Chapter 6)	Birds (Chapter 7)
	Cellular composition of bridging white matter (commissures, callosum).		Many TH up-regulated genes directly linked with metamorphic events have been studied, including, but not limited to; stromelysin-3, TH/bZIP, and TRβ.	
Liver				
Genes	Malic Enzyme		**Potential genes found in late response to TH** - Carbamyl-phosphate synthetase I, arginosuccinate synthase and lyase, arginase, N-CAM, albumin	Malic enzyme gene expression/protein production in avian embryos (not well established)
	Alpha GPD		Type II and III Deiodinase	
	Type I Deiodinase		**Metabolic changes** - shift from ammonotelism to ureotelism	
	Thyroxin-binding Globulin (TBG)			
Heart				
Genes	SERCA-1		—	
	SERCA-2		—	
	MHC		**MHC** - In *X. laevis*, class I antigens are virtually absent from larval tissues until metamorphic climax (Rollins-Smith et al., 1997a	
Cardiovascular function	Heart rate		—	
	Blood pressure		—	
Tail				
Genes			Many TH up-regulated genes directly linked with metamorphic events have been studied	
Other Endpoints				
		Flounder stomach formation (gastric glands)	CRF, which is the amphibian TRH	

Specific End Points	Mammals (Chapter 3)	Fish (Chapter 5)	Amphibians (Chapter 6)	Birds (Chapter 7)
			Genes in the metamorphosing tail (Xenopus laevis).	

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APPENDIX A

HYPOTHALAMIC-PITUITARY-THYROID RESPONSES TO THYROID TOXICANTS PERCHLORATE, PROPYLTHIOURACIL, AND POLYCHLORINATED BIPHENYLS (ZOELLER 2005)

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Hypothalamic-Pituitary-Thyroid Responses to Thyroid Toxicants Perchlorate, Propylthiouracil and Polychlorinated Biphenyls

by

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for

Work Assignment 5-5, Task 13

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EXECUTIVE SUMMARY

To compare the effects of chemicals that act through different modes of action on the HPT axis, we have performed an analysis of three thyroid toxicants which the literature was complete enough to include in this analysis. Toxicants included ammonium perchlorate, which inhibits iodide uptake by the sodium/iodide symporter, propylthiouracil (PTU), which inhibits thyroperoxidase and the type 1 deiodinase, and polychlorinated biphenyls (PCBs), which increase liver glucuronidase enzymes and displace T_4 from transthyretin *in vitro*. To the extent possible, we have evaluated both the qualitative and quantitative relationships between toxicant dose, serum hormone levels, thyroid weight and thyroid histopathology.

Qualitative analysis of these studies indicates that chemicals acting on the HPT axis by different mechanisms exert a subtly different pattern of effects on measures of thyroid function. Perchlorate tends to affect T_3 at doses lower than those that affect other hormonal endpoints. However, thyroid histology appears to be more sensitive to perchlorate exposure overall, but this conclusion is heavily influenced by a single study. Some of the dose-response profiles of perchlorate on these measures were somewhat unexpected – especially the case of an "inverted U" dose-response of T_4. Measures of HPT axis function were most consistent in response to PTU exposure–serum T_4, T_3, TSH and thyroid weight and histopathology responded to similar doses of PTU. In contrast, the HPT axis responded most inconsistently to PCB exposure. Although serum total T_4 was always reduced by PCBs, serum total T_3, TSH and thyroid weight were not always affected. In part, this could be due to difference in the PCBs being used in the different studies. Differences among studies indicate that gender, duration of treatment and age of exposure are important variables associated with the response profile.

The relative sensitivities of four continuous variables (total T_3 (TT3), TT4, TSH, and thyroid weight), and one non-continuous variable (thyroid histology) were compared based on these data. Sensitivity of an activity measure was expressed in terms of steepness of the dose response, as indicated by the slope of the dose response relation. The activity measures were ranked based on the lower bounds of the 95 percent confidence intervals on absolute slope.

For perchlorate the analysis indicated that hypertrophy was relatively sensitive compared to the continuous measures, and that TT3 and TT4 were relatively less sensitive than TSH and thyroid weight. The relatively high sensitivity of hypertrophy was heavily influenced by the results from a single study however, York 2001. For PTU and PCBs the inference sensitivity for comparisons among measures was lower than for perchlorate because there was less data available. For PTU there was no indication of sensitivity differences among measures. For PCBs there was no hypertrophy data, and total T_4 had relatively higher sensitivity than the other continuous measures.

A sensitivity analysis was carried out in which an alternative method was used to calculate the slopes of the continuous activity measures. This was done to determine whether the relatively high average sensitivity observed for hypertrophy in the perchlorate studies would still hold. For perchlorate the hypertrophy average sensitivity was still relatively high compared to the average sensitivities of the continuous measures. This again was in large part due to the sensitivity ranks in the York 2001 study. The relative sensitivities of the four continuous measures differed somewhat from that determined with the logarithmic analysis, in that they were all equivalent. For PTU, the relative sensitivities agreed with those determined with the original analysis. The confidence bands were wider than those for perchlorate and overlapped across all the measures. For the PCBs the confidence band for thyroid weight was higher than those for the other measures, implying that this endpoint was more variable and perhaps less sensitive than the hormone measurements.

In summary the relation between the sensitivities of the continuous response measures and that of the ordinal response measure agreed between both sets of calculations. The relations among the continuous measures differed between the two sets of calculations. Since the second set of calculations assumed constant standard deviation across doses, which was sometimes violated, the relations among the continuous measures based on the first analysis may be more appropriate.

INTRODUCTION

Many compounds are identified as being thyrotoxic (Brucker-Davis, 1998), defined by their ability to alter circulating levels of thyroid hormone. As described by Devito *et al.* (DeVito *et al.*, 1999), xenobiotic effects on hormone levels (T_3, T_4, TSH) and thyroid weight and histology are currently the most important endpoints of thyroid toxicity. However, it is not clear that chemicals acting through different modes of action to reduce serum hormone levels all exert the same pattern of effects on the profile of hormone changes and on thyroid weight and histology. Therefore, the goal of the current analysis was to determine whether this idealized model of HPT axis function is observed across chemicals that have different modes of action, and to determine which of the endpoints of thyroid function is most sensitive to thyroid toxicants. For example, while it is predicted that thyroid weight and histology would change in response to changes in serum TSH, it is possible that the technical features of the TSH assay make it more difficult to detect a change than are changes in thyroid weight. Moreover, because thyroid hormones and TSH exhibit various rhythms in the blood, it is possible that single point estimates of hormone levels are quite variable and that thyroid weight/histology represents a sensitive measure of changes in TSH integrated over the duration of toxicant exposure.

As reviewed in Chapter 3, thyroid hormone exerts a negative feedback effect on both the hypothalamic release of TRH and the pituitary release of TSH. Thus, in principle, as serum T_4 levels decline, serum TSH should increase. Because TSH exerts a trophic effect on the thyroid gland, thyroid weight should increase with increasing TSH levels, and thyroid histology should be altered. The response of serum T_3 to altered thyroid function is more difficult to predict considering that nearly 80% of serum T_3 is derived from peripheral deiodination of T_4. Therefore, changes in serum T_3 may be more an indicator of changes in peripheral metabolism of T_4 than of TSH action on the thyroid gland itself. However, this becomes complicated in cases such as low iodide because T_3 becomes the dominant hormone formed by the thyroid gland; thus, the ratio of T_4 to T_3 is controlled by several processes that may be influenced by environmental factors and changes in serum T_3 may or may not parallel changes in serum T_4.

To determine the degree to which different thyroid toxicants exert these idealized effects on the HPT axis, we have performed an analysis of three thyroid toxicants, chosen for their different modes of action on the HPT axis. Toxicants included ammonium perchlorate, which inhibits iodide uptake by the sodium/iodide symporter (Wolff, 1998), propylthiouracil (PTU), which inhibits thyroperoxidase (Nagasaka and Hidaka, 1976; Sugawara, 1985) and the type 1 deiodinase, and polychlorinated biphenyls (PCBs), which increase liver UDPGT enzymes and displace T_4 from transthyretin *in vitro* (Brouwer *et al.*, 1998). To the extent possible, we have evaluated the quantitative relationship between toxicant, serum hormone levels, thyroid weight and thyroid histopathology.

METHODS

Identification of Reports. To identify published reports of toxicant effects on the HPT axis, we searched the MEDLINE database using EndNote™ 8.0 and key words ("toxicant" x "thyroid weight" x "rat"). Initial searches revealed that other key words (e.g., "T_4", "thyroxine", "T_3", "TSH") obtained a greater number of "hits", but fewer studies that contained all of the desired endpoints (T_4, T_3, TSH, thyroid weight and thyroid histopathology). All abstracts were inspected for the inclusion of the desired endpoints and all reports containing these endpoints were obtained. A few reports are included that lack specific endpoints (either TSH or thyroid weight) because the number of reports that contain all of these endpoints was surprisingly low. Initially, authors were contacted to supply their original data to improve the statistical analysis, but no authors complied. Papers were targeted that used adult rats, with the exceptions of a two-generation study and a study of cross-fostered rats.

Data Extraction. Data were extracted from the reports as follows. First, numerical data found in tables were manually transferred to a Microsoft Excel file and proofread. Each file contains all of the data extracted for a particular chemical. Serum T_3 and T_4 were converted to nM so that these values are more easily compared across studies. Graphical data were converted to numerical data using a Mitutoyo NTD12 6-inch digital caliper to physically estimate values represented on graphs. To accomplish this, the scale of each graph was first measured to convert from millimeters (height of the histogram or line drawing) to the unit being reported by the graph. Converted values (e.g., T_3 in ng/dL, T_4 in µg/dL, etc) were transferred to the Excel spreadsheet. In all cases, error (SD or SEM) was included among the data extracted, and the coefficient of variation was calculated.

Data Analysis. Data were first evaluated qualitatively for evidence of an "idealized" response to toxicant, and described qualitatively in this report. In addition, the extracted data were sent to Battelle and evaluated quantitatively as described by Feder and Feng (Feder and Feng, 2005), which is provided as Appendix B to the DRP.

RESULTS

I. PERCHLORATE

Perchlorate was chosen in this review because it is a known anti-thyroid agent (Wolff, 1998) and because there are a number of recent studies evaluating the toxicity of perchlorate on the HPT axis in rats. Perchlorate is known to inhibit iodide uptake into the thyroid gland by the sodium/iodide symporter (NIS) (Carrasco, 2000). Thus, in sufficient doses, perchlorate is known to inhibit iodide uptake to such an extent that circulating levels of thyroid hormones (T_4 and T_3), decline. The search terms "Perchlorate" x "Thyroid weight" x "rat" identified 22 research articles in MEDLINE. These references are included in Appendix A.1. Of these 22 reports, 5 were chosen for analysis because they contained measures of serum T_3, T_4, TSH, thyroid weight and histopathology. These reports (Hiasa *et al.*, 1987; Siglin *et al.*, 2000; York *et al.*, 2001; Mahle *et al.*, 2003; York *et al.*, 2003; York *et al.*, 2004) were then used to extract data and analyze the relationships among hormone levels, thyroid weight and histology, and toxicant dose. The extracted data are provided in the report by Feder and Feng (Appendix B). The citations for the reports themselves are provided in Appendix A.3.

A) ***York, R. G., Brown, W. R., Girard, M. F., and Dollarhide, J. S. (2001). Two-generation reproduction study of ammonium perchlorate in drinking water in rats evaluates thyroid toxicity. Int J Toxicol 20, 183-197.***

York *et al.* (York *et al.*, 2001) published the results of a two-generation reproduction study of the effects of ammonium perchlorate in drinking water in Sprague-Dawley rats. The parental generation of

animals (30/group) were exposed to perchlorate in drinking water beginning two weeks prior to cohabitation and then throughout gestation and lactation at doses targeted to deliver 0 mg/kg-day, 0.3 mg/kg-day, 3.0 mg/kg-day, and 30 mg/kg-day. The concentration of perchlorate in drinking water was calibrated to deliver these doses based on current body weight and water consumption measured the previous week. Water consumption was estimated based on weight of the water bottle. The F1 generation rats were given the same ammonium perchlorate doses as their respective P1 generation sires and dams beginning at weaning (they were exposed to perchlorate lactationally prior to this) and continuing through the day of sacrifice. Standard reproductive parameters were evaluated; blood was collected for determination of serum thyroid-stimulating hormone (TSH), total triiodothyronine (T3), and total thyroxine (T4) levels. Histopathological examination was conducted on major tissues, including the thyroid.

Parental adults. Serum total T_3 was significantly elevated in the P1 males by 3.0 and 30.0 mg/kg-day perchlorate, but was unaffected by perchlorate in females. In contrast, serum total T_4 levels were decreased in males treated with 30 mg/kg-day and in females treated with 0.3 and 3.0 mg/kg-day only. Similarly, serum TSH was affected in males only by the highest dose of perchlorate (30 mg/kg-day) and was unaffected in females. Both absolute thyroid weight and measures of thyroid hypertrophy/hyperplasia were increased in males and females treated with the highest dose of perchlorate.

The observed effects of perchlorate on serum total T_3 are difficult to reconcile based on what we know about the regulation of serum thyroid hormones. Specifically, serum total T_3 is increased in animals given perchlorate at 0.3 and 3.0 mg/kg-day, but there is no concomitant decrease in serum total T_4 as would be predicted if the animals were deprived of iodide. Thus, the relationship between T_3 and T_4 are not predictable. In contrast, the relationship between serum T_4, TSH and thyroid measurements is generally consistent. Serum TSH increases when serum total T_4 declines and this is associated with changes in thyroid histology.

In contrast to the P1 adults, the F1 adults exhibited a different thyroidal response to perchlorate. Serum total T_3 was unaffected by perchlorate in both males and females. Serum total T_4 in males was elevated by perchlorate at 0.3 and 3.0 mg/kg-day and then decreased by perchlorate at 30.0 mg/kg-day; serum TSH tended to decrease at the lower doses but was significantly elevated at 30 mg/kg-day. In females, serum total T_4 was not affected, but serum TSH was elevated at the highest perchlorate dose. Interestingly, thyroid weight was affected by perchlorate in males at 3.0 and 30.0 mg/kg-day and was affected by all perchlorate doses in females. Measures of thyroid histology were significantly affected by perchlorate in both males and females only at the highest dose of perchlorate.

The hormonal and thyroidal response to perchlorate in the F1 adults is difficult to fully explain using an idealized model of responses of the HPT axis. The "inverted U" dose response of perchlorate on T_4 is somewhat reconcilable with the literature. Several investigators have reported an increase in serum T_4 following perchlorate exposure in humans (Crump *et al.*, 2000; Braverman *et al.*, 2005) and in animals (Thuett *et al.*, 2002). Thus, the increase in serum total T_4 reported by York *et al.* (York *et al.*, 2001) at doses of 0.3 and 3.0 mg/kg-day is not unprecedented. Moreover, the following decrease in serum total T_4 at 30.0 mg/kg-day is consistent with the mode of action of perchlorate on the thyroid gland. No one has proposed an explanation for the association of perchlorate exposure and elevated serum T_4. York *et al.* (York *et al.*, 2001) conclude that it is not treatment-related, as does Crump et al. (Crump *et al.*, 2000). Braverman *et al.* (Braverman *et al.*, 2005) suggests that the increased serum T_4 may be related to a decrease in thyroidal iodide content causing an increase in thyroidal response to TSH.

Serum thyroid hormones were virtually unaffected by perchlorate exposure in both the F1 and F2 pups. These pups were evaluated for hormone measurements at the time of weaning (postnatal day 21). Thyroid weight was significantly increased at the highest dose level of perchlorate.

B) York, R. G., Funk, K. A., Girard, M. F., Mattie, D., and Strawson, J. E. (2003). Oral (drinking water) developmental toxicity study of ammonium perchlorate in Sprague-Dawley rats. Int J Toxicol 22, 453-464.

This was a developmental toxicity study in which adult male and female Sprague-Dawley rats were exposed to ammonium perchlorate in their drinking water at doses of 0.0, 0.01, 0.1, 1.0, and 30.0 mg/kg-day beginning 14 days before insemination and continuing through sacrifice on gestation day 21. Adult females and their fetuses were evaluated for clinical features as well as for serum hormones (total T_3 and T_4, TSH) and thyroid histology.

Serum T_4 was reduced in dams exposed to all doses of perchlorate, and serum TSH was elevated; thyroid weight was elevated only in the highest dose-group. Likewise, measures of thyroid histology (hypertrophy) were significantly affected only by the highest dose of perchlorate.

In contrast, total T_4 was significantly reduced in pooled fetal serum (G21) only in those fetuses exposed to the highest dose of perchlorate, and serum TSH was elevated in fetal serum following exposure to 1.0 and 30.0 mg/kg-day. Serum total T_3 was reduced by all doses of perchlorate. No measures of thyroid histology were affected by perchlorate.

A significant weakness in this study was that control levels of T_4 in both dams and fetuses were below the lowest standard used in their RIA. Thus, these measures are unreliable. Despite this, dams exhibited decreased serum total T_4 in all perchlorate-exposed groups, and serum TSH was elevated. Pooled serum was used to measure serum hormone levels in the fetus. While this is especially important for serum TSH, which uses a serum volume of about 200 µl, this pooling may well blunt the ability to detect effects of perchlorate exposure. The no-observable-adverse-effect-level (NOAEL) in the fetus was considered to be 1.0 mg/kg-day; developmental delays in ossification of fetal skeleton were found at 30.0 mg/kg-day.

C) York, R. G., Barnett, J., Jr., Brown, W. R., Garman, R. H., Mattie, D. R., and Dodd, D. (2004). A rat neurodevelopmental evaluation of offspring, including evaluation of adult and neonatal thyroid, from mothers treated with ammonium perchlorate in drinking water. Int J Toxicol 23, 191-214.

Mated Sprague-Dawley rats were exposed to ammonium perchlorate in drinking water (25/group) at concentrations of 0.0, 0.1, 1.0, 3.0, and 10.0 mg/kg-day. The duration of exposure extended from the day of mating to postnatal day (day of lactation) 10. One pup/sex/litter/treatment group was assigned to a variety of endpoints including serum hormone levels, thyroid weight and histology, juvenile brain weights, morphometry and neuropathology, behavioral assays and adult regional brain weights, morphometry and neuropathology.

In serum pooled from pups culled on postnatal day 5, total T_4 was significantly reduced in animals derived from dams treated with 3.0 and 10.0 mg/kg-day. This was also true for total T_3, but the degree to which total T_3 was reduced was considerable (nearly 50%). Serum TSH in this pooled serum was significantly elevated only by the highest dose of perchlorate (10 mg/kg-day). Measures of thyroid histology were affected in these pups generally by 3.0 and 10.0 mg/kg-day.

D) Siglin, J. C., Mattie, D. R., Dodd, D. E., Hildebrandt, P. K., and Baker, W. H. (2000). A 90-day drinking water toxicity study in rats of the environmental contaminant ammonium perchlorate. Toxicol Sci 57, 61-74.

Adult male and female Sprague-Dawley rats were continuously exposed to ammonium perchlorate in drinking water for 14 and 90 days. Dosage levels included 0.0, 0.01, 0.05, 0.2, 1.0, and 10.0 mg/kg-day.

Among other endpoints, serum hormone levels (T_3, T_4, TSH) and thyroid histology, were measured at these times.

Serum total T_3 was decreased in males exposed to all doses of perchlorate at both 14 and 90 days of exposure, whereas serum total T_4 was reduced by 10 mg/kg-day at 14 days and all doses of perchlorate at 90 days. Serum TSH was elevated in males at 0.2 mg/kg-day and above at both 14 and 90 days of exposure. Perchlorate effects on serum total T_3 and TSH were reversed 30 days following the cessation of 90 days treatment in males, but this was not the case for serum total T_4 which remained reduced in all dose groups evaluated (0.05 mg/kg-day and above).

In contrast, females exhibited a decline in serum total T_3 by perchlorate only following 90 days of exposure. Serum total T_4 was reduced by 10 mg/kg-day at 14 days, but by all exposure levels at 90 days. Paradoxically, serum TSH was elevated in animals exposed to 0.05 mg/kg-day and above at 14 days, but not in any perchlorate-exposed groups at 90 days.

Thyroid weight was affected by the highest dose of perchlorate (10 mg/kg-day) at both 14 and 90 days in males, but only at 90 days in females. No significant effects on morphometric measurements of thyroid gland histology were reported.

Taken together, these data are somewhat difficult to reconcile with an idealized response of the HPT axis to iodide uptake inhibition. First in males, serum TSH levels increase at 14 days despite a failure for T_4 levels to decline. Serum TSH is more sensitive to T_4 than to T_3 according to some reports (Schneider *et al.*, 2001). Moreover, despite the increase in serum TSH, thyroid weight and measures of thyroid histology were not affected. Likewise in females, serum TSH was elevated in animals in which serum T_4 was not affected; serum T_3 was decreased in animals in which serum T_4 were not affected, and measures of thyroid histology were not affected despite an increase in serum TSH. Thus, the relationship between iodide uptake inhibition and measures of HPT axis function were not completely reconcilable with an idealized model, leading to complications in the interpretation of these results.

E) Mahle, D. A., Yu, K. O., Narayanan, L., Mattie, D. R., and Fisher, J. W. (2003). Changes in cross-fostered Sprague-Dawley rat litters exposed to perchlorate. Int J Toxicol 22, 87-94.

Pregnant Sprague-Dawley rats were given either untreated or perchlorate (1 mg/kg-day) treated drinking water beginning on gestation day 2. One set of control and exposed dams was sacrificed on gestation day 20. The litters from the second set of control and exposed dams were crossed immediately after parturition and were sacrificed at postnatal day 10. Dam serum and thyroid, pooled fetal sera, and male and female pup sera were collected and analyzed for perchlorate, thyroid-stimulating hormone (TSH), triiodothyronine (T3), and thyroxine (T4). Control pups receiving perchlorate through lactation had serum levels at postnatal day 10 of 0.54 μg/ml and 0.56 *1*g/ml for male and female pups, respectively, whereas exposed fetuses had serum perchlorate levels of 0.38±0.04 μg/ml.

Serum total T_4 was decreased by perchlorate in dams at GD20 and at PND10 if perchlorate exposure had continued. Serum total T_4 was also reduced by perchlorate exposure in pups at GD 20, and in females at PND 10 that were exposed during lactation. In contrast, male pups did not exhibit an effect of perchlorate exposure on serum total T_4. Likewise, serum TSH was elevated by treatment in dams at GD20 and at PND 10 if perchlorate exposure was continuous from GD2. Moreover, serum TSH was elevated in pups at GD20, and at PND 10 if perchlorate exposure occurred lactationally (no gender difference). Serum total T_3 were essentially unaffected by treatment.

While these findings are consistent with an idealized model of iodide uptake inhibition on the HPT axis, they are not consistent with findings reviewed above. In both Siglin et al. and York et al., serum total

T_3 was affected at doses of perchlorate exposure below that which affected serum total T_4. This was not the case in Mahle et al. However, these data are internally consistent and are consistent with an conventional model of the effects of iodide uptake inhibition on the HPT axis.

F) Hiasa, Y., Kitahori, Y., Kato, Y., Ohshima, M., Konishi, N., Shimoyama, T., Sakaguchi, Y., Hashimoto, H., Minami, S., and Murata, Y. (1987). Potassium perchlorate, potassium iodide, and propylthiouracil: promoting effect on the development of thyroid tumors in rats treated with N-bis(2-hydroxypropyl)- nitrosamine. Jpn J Cancer Res 78, 1335-1340.

Adult male Wistar rats were exposed to control or perchlorate (100 ppm) infused feed for 19 weeks. Neither serum total T_4 nor serum total T_3 was affected by perchlorate treatment, but serum TSH was elevated. Thyroid weight was unaffected. Considering that, in general (personal observation), the intra- and inter-assay coefficient of variation is greater for peptide assays than for T_4 or T_3, it is difficult to reconcile these findings either from the point of view of an idealized response of the HPT axis to iodide uptake inhibition, or from the point of view of the technical characteristics of these assays.

G) Feder, P. I., and Feng, J. (2005). Draft report on comparison of thyroid activity measures across data sets. EPA Contract No. 68-W-01-023, WA No. 5-3.

This quantitative analysis indicated that the thyroid morphometry data are the most sensitive indicators of perchlorate exposure among all studies. However, this conclusion is heavily influenced quantitatively by a single study – that of York *et al.* (2001). Serum TSH is the next most sensitive indicator of perchlorate exposure, which is consistent with the finding that serum TSH responds logarithmically to changes in serum total T_4.

General Conclusions. There are several conclusions that can be drawn from this analysis. First, the effect of perchlorate on serum thyroid hormones and thyroid gland weight differ among studies; the duration of exposure, age of exposure and gender, are likely to be important variables. The duration of exposure is likely to be important in the case of perchlorate because the thyroid gland contains a fairly large storage pool of thyroid hormone in the colloid that may have to be exhausted before thyroid hormone levels decline. This is consistent with the report of Siglin et al. in which lower doses of perchlorate affected thyroid hormone levels at 90 days of exposure compared to 14 days of exposure. In addition, the age and gender of the animal also appears to be important. Pups (P5) exhibited a tighter relationship between dose of perchlorate and various endpoints of thyroid function (York *et al.*, 2004). This tighter relationship may occur because pups have less hormone stored in their thyroid glands and, therefore, an inhibition of thyroid hormone synthesis may result in lower hormone levels more rapidly.

The second conclusion is that the HPT response to perchlorate is not quite the anticipated response. For example, in the Siglin study, serum T_3 was the most sensitive endpoint to perchlorate exposure, and thyroid weight was the least sensitive. This is difficult to reconcile because nearly 80% of circulating T_3 is derived from peripheral deiodination of T_4, yet T_3 was lowered while T_4 levels are normal. In addition, serum TSH levels were not significantly elevated by doses of perchlorate that significantly lowered serum T_4 either in the Siglin or York studies. Although thyroid weight was not affected by perchlorate in the York study, various features of thyroid histology were affected. Specifically, thyroid follicle luminal area decreased with increasing dose of perchlorate, and was the most sensitive endpoint of perchlorate exposure, perhaps because serum TSH was not altered. That is, perchlorate exposure inhibited thyroid hormone synthesis, which required that colloid be removed in the absence of additional stimulation by TSH.

The quantitative relationship between variables analyzed by the global model of Feder and Feng (Appendix B) indicated that, for perchlorate overall, measures of hypertrophy were relatively sensitive

compared to hormone levels, and that TT3 and TT4 were relatively less sensitive than TSH and thyroid weight. The relatively high sensitivity of hypertrophy was heavily influenced by the results from a single study, that of York 2001. Quantitative differences among studies in the dose-response curves and in the observed relationship among hormones and thyroid weight/histology (i.e., departure from an idealized relationship) may be related to the mechanism of thyroid toxicity (i.e., NIS inhibition) and/or an interaction with dietary components such as vitamins (Mutaku *et al.*, 1998), dietary iodide content, or inclusion of soy-based protein (Divi and Doerge, 1996).

II. PROPYLTHIOURACIL (PTU)

Propylthiouracil was chosen for this analysis because it is a well-studied anti-thyroidal agent (Cooper *et al.*, 1983). PTU can inhibit the ability of thyroperoxidase (TPO) to activate iodine and transfer it to thyroglobulin (Davidson *et al.*, 1978). In addition, PTU inhibits the activity of the type 1 deiodinase (D1, (Goswami and Rosenberg, 1986)). The search terms "PTU" x "thyroid weight" x "rat" identified 134 reports in MEDLINE (see Appendix A.2). Of these 134 reports, 4 were chosen for analysis because they contained measures of serum T_3, T_4, TSH, thyroid weight and thyroid histopathology. These references (Hood *et al.*, 1999; O'Connor *et al.*, 1999; O'Connor *et al.*, 2002b; Cho *et al.*, 2003; Mellert *et al.*, 2003) were then used to extract data and analyze the relationships among hormone levels and toxicant dose. The extracted data are provided in Feder and Feng (Appendix B).

A) Mellert, W., Deckardt, K., Walter, J., Gfatter, S., and van Ravenzwaay, B. (2003). Detection of endocrine-modulating effects of the antithyroid acting drug 6-propyl-2-thiouracil in rats, based on the "Enhanced OECD Test Guideline 407". Regul Toxicol Pharmacol 38, 368-377.

Propylthiouracil (PTU) was administered to male and female Wistar rats (42-44 days old) at 0, 0.1, 1, or 10 mg/kg body weight for 28 days according to the draft protocol of the ''Enhanced OECD Test Guideline 407'' (enhanced TG 407). The study was conducted with two identical subsets of five animals per sex and dose. At the time of sacrifice, all females were in the diestrus stage of estrous. Doses of PTU were administered by gavage daily (dissolved in water) for 28 days (study days 0 to day 27). Estrous cycle determination started on day 21 of the administration period. On days 22 and 24 (males subsets A and B) and 23 and 25 (females subsets A and B), respectively, a functional observation battery and the measurement of motor activity were conducted. One day after the last administration of the test substance and last weighing, blood parameters were examined. All males were sacrificed on day 28 of the study, the females—dependent on the estrus cycle— were necropsied on day 31 at the latest. Serum total T_3 was reduced in males and females exposed to 10 mg/kg-day. In contrast, serum total T_4 was reduced in both males and females exposed to 1 and 10 mg/kg-day. Serum TSH was increased in both males and females exposed to 1 or 10 mg/kg-day and in males exposed to 0.1 mg/kg-day in one of the two subsets.

Table 5
Significant treatment-related effects (*$P < 0.05$; **$P < 0.01$) dependent on PTU concentration (mg/kg)

	Males			Females		
	A	B	A + B	A	B	A + B
T3	**10****	**10****	**10****	**10****	**10****	**10****
T4	**1****	**1****	**1****	1**	1*	0.1*
TSH	0.1*	1*	1**	1**	10**	1**
Thyroid weight	**1****	**1****	**1****	0.1*	1**	1**
Pituitary weight	10*	10**	10**	1*	10*	1*

Note. Bold numerals indicate statistical homogenous results in evaluating subsets A, B, and A + B.

In general, these authors found that serum T_4 was more sensitive to PTU treatment than was T_3 (1 vs 10 mg/kg-day; See Table 5 of Mellert et al.). TSH and thyroid weight were also affected by 1 mg/kg-day.

These findings are quite consistent with an idealized model of the HPT axis in which T_4 levels decline, followed by serum T_3, leading to an increase in serum TSH and thyroid weight.

B) O'Connor, J. C., Frame, S. R., Davis, L. G., and Cook, J. C. (1999). Detection of thyroid toxicants in a tier I screening battery and alterations in thyroid endpoints over 28 days of exposure. Toxicol Sci 51, 54-70.

Male Sprague-Dawley rats were treated for 15 days with either Phenobarbital (5, 25, 50 or 100 mg/kg-day) or PTU (0.025, 0.25, 2.0 or 10 mg/kg-day). Drugs were dissolved in 0.25% methylcellulose and injected intraperitoneally. Serum hormones and thyroid weight was then evaluated.

In females, PTU at 10 mg/kg-day significantly decreased serum T_3 and T_4, and TSH was elevated. In males, PTU decreased serum total T_3 and T_4 and increased TSH levels at concentrations of 0.025 mg/kg-day and above. Thyroid weight and measure of cell proliferation were significantly affected by PTU exposure at all doses in males, but only at the highest dose in females. Caloric restriction, a potential confounder in this assay, caused a reduction in serum TSH, T_4 and T_3; while thyroid weight was unaffected.

These findings indicate that males are more sensitive to PTU than are females, and that thyroid weight and measures of thyroid histopathology were more sensitive indicators of PTU effects than are hormone levels. Moreover, these data demonstrated that caloric restriction produced a central inhibition of the HPT axis (i.e., reduction in serum TSH and T_4), consistent with many studies (Rondeel *et al.*, 1992; Ueta *et al.*, 1995; van Haasteren *et al.*, 1995; van Haasteren *et al.*, 1996; Zimmermann-Belsing *et al.*, 2003; Lechan and Fekete, 2004). However, the issue of caloric restriction will not be developed in the current report. Rather, it is an issue that needs to be carefully controlled in studies in which animals restrict their caloric intake.

C) O'Connor, J. C., Frame, S. R., and Ladics, G. S. (2002). Evaluation of a 15-day screening assay using intact male rats for identifying steroid biosynthesis inhibitors and thyroid modulators. Toxicol Sci 69, 79-91.

Male Sprague-Dawley rats were treated by gavage for 15 days with either Phenobarbital (5, 25, 50 or 100 mg/kg-day) or PTU (0, 0.1, 1.0, 10.0, and 20.0 mg/kg-day). Serum hormones and thyroid weight was then evaluated.

Serum total T_3 was significantly decreased by exposure to PTU doses of 1.0 mg/kg-day and above. Serum total T_4 was significantly decreased by the same doses, and serum TSH was elevated similarly. Thyroid weight was significantly increased by exposure to doses of PTU of 0.1 mg/kg-day and above, and "microscopic" changes in thyroid histology were consistent with this.

Therefore, thyroid weight and histopathology were the most sensitive indicators of thyroid toxicity to PTU in this study. Likewise, serum T_4, T_3, and TSH all exhibited the same sensitivity to PTU.

D) Hood, A., Liu, Y. P., Gattone, V. H., 2nd, and Klaassen, C. D. (1999). Sensitivity of thyroid gland growth to thyroid stimulating hormone (TSH) in rats treated with antithyroid drugs. Toxicol Sci 49, 263-271.

Adult male Sprague-Dawley rats were fed a diet containing PTU at concentrations of 0, 1, 3, 10, 30, 100 or 300 ppm). Measures of thyroid hormones and thyroid weight/histology were obtained 21 days later. Serum total T_3 was significantly reduced by exposure to PTU concentrations of 3 ppm and higher following the first day of treatment, but thereafter, only concentrations of 10 ppm and higher caused a reduction in serum total T_3. Likewise, serum total T_4 was reduced by exposure to PTU at concentrations of

10 ppm and higher throughout the 21 days. Serum TSH was elevated in animals exposed to PTU at concentrations of 10 ppm and higher following at least 7 days of treatment. Interestingly, TSH levels declined to control values in animals exposed to PTU at 3 ppm at 21 days. At 21 days of exposure, serum total and free T_4 and serum total and free T_3 were significantly reduced by exposure to PTU at concentrations of 10 ppm and higher. At 21 days, thyroid weight was significantly elevated in animals exposed to PTU at concentrations of 10 ppm and higher. This was associated with a significant increase in "labeling index" (i.e., cell proliferation) in animals exposed to PTU at concentrations of 30 ppm and higher.

The results of this study are quite consistent with the idealized model of HPT responses to thyroid toxicity. Serum T_4, TSH and thyroid weight appear to be causally linked. In this report, serum T_3 follows serum T_4.

E) Cho, S. D., Kim, J. H., Kim, D. Y., Lee, Y. S., and Kang, K. S. (2003). Pre- validation study for OECD enhanced test guideline 407 protocol by gavage for 4 weeks using propylthiouracil and tamoxifen. Toxicol Lett 144, 195-204.

Adult male and female Sprague-Dawley rats were exposed to a variety of doses of PTU, dissolved in corn oil, for 28 days. Doses included 0.0, 0.1, 1.0, and 10.0 mg/kg-day, given by daily gavage. Serum total T_3 was decreased only in males exposed to 10.0 mg/kg-day; not in females. Likewise, serum total T_4 was affected only by treatment with the highest dose of PTU in males, not in females. Serum TSH was unaffected by treatment in males, but was significantly elevated in females exposed to the highest concentration of PTU. All males exposed to PTU concentrations of 1.0 mg/kg-day and above exhibited histological changes in the pituitary gland. Females did not respond as uniformly as did males.

This particular study is difficult to reconcile with an idealized model of HPT axis responses to PTU, other than to surmise that the RIAs used in this study were somehow compromised. It is difficult to reconcile differences in thyroid histology associated with PTU exposure in the absence of a reduction in serum T_4 and an increase in serum TSH.

F) Feder, P. I., and Feng, J. (2005). Draft report on comparison of thyroid activity measures across data sets. EPA Contract No. 68-W-01-023, WA No. 5-3.

Quantitative analysis of these data by Feder and Feng indicated that there was no indication of sensitivity differences among measures of serum thyroid hormones, TSH and thyroid weight/histology. This analysis is consistent with several of the studies qualitatively reviewed here. However, it also appears important to entertain the possibility that there are technical issues governing the use of RIA that need to be defined across laboratories so that all studies would produce comparable results.

III. POLYCHLORINATED BIPHENYLS (PCBs)

Polychlorinated biphenyls (PCBs) were chosen as a test case for this analysis because they are well-studied anti-thyroidal agents (Zoeller, 2001). PCBs exert direct effects on the thyroid gland, on the ability of T_4 to bind to serum transthyretin (TTR), and on liver glucuronidase expression (Brouwer *et al.*, 1998). Although there are 209 different PCB congeners, based on the number and position of chlorine substitutions, all are known to reduce circulating levels of T_4. The search terms "Polychlorinated Biphenyl" x "Thyroid weight" x "rat" identified 30 reports in MEDLINE (see Appendix A.3). These studies did not contain reports including all of the study parameters (serum T_3, T_4, TSH, thyroid weight and thyroid histopathology). Therefore, we chose reports that had the greatest number of these parameters, though we were not able to include measure of thyroid histopathology in our analysis. In addition, these references did not all study the same PCB congeners or mixtures. Although this weakens our ability to

interpret the current data set, PCBs are one of the best-studied thyroid toxicants, and the data presented here are useful in testing whether thyroid toxicants produce effects on the HPT axis that are predictable, regardless of the mode of action. These references (Kodavanti *et al.*, 1998; Desaulniers *et al.*, 1999; Meerts *et al.*, 2002; Kato *et al.*, 2003; Bowers *et al.*, 2004; Meerts *et al.*, 2004; Vansell *et al.*, 2004) were then used to extract data and analyze the relationships among hormone levels and toxicant dose. The extracted data are provided in Feder and Feng (Feder and Feng, 2005).

A) Bowers, W. J., Nakai, J. S., Chu, I., Wada, M., Moir, D., Yagminas, A., Gill, S., Pulido, O., and Meuller, R. (2004). Early developmental neurotoxicity of a PCB/Organochlorine mixture in rodents after gestational and lactational exposure. Toxicol Sci 77, 51-62.

The developmental and neurobehavioral effects of gestational and lactational exposure to a mixture of 14 polychlorinated biphenyls (PCBs) and 11 organochlorine pesticides was examined and compared against the commercial PCB mixture Aroclor 1254. Only data derived from effects of Aroclor are extracted for comparison with other studies. Pregnant Sprague-Dawley rats were dosed orally by daily feeding animals a Teddy Graham cookie dosed with either 0.0 or 15 mg/kg of Aroclor 1254 from gestation day (GD) 1 to postnatal day (PND) 23. Aroclor 1254 produced smaller but persistent decreases in offspring weight without affecting maternal weight or offspring mortality.

In both dams and pups, A1254 exposure significantly decreased serum total T_4 and serum total T_3, but did not affect serum TSH. Morphormetric analysis of thyroid histology also revealed that pups exposed to A1254 exhibited a significant decrease in colloid area and the proportion of follicles containing a colloid greater than 1000 mm^2. In addition, the measure of "roundness" was significantly increased.

These data indicate that PCBs (A1254) significantly decrease the circulating concentrations of both T_4 and T_3, but do not affect serum TSH. However, the morphometric analysis of thyroid histology indicates that PCB exposure significantly affects colloid size – perhaps indicating a significant decrease in the amount of stored hormone.

B) Desaulniers, D., Leingartner, K., Wade, M., Fintelman, E., Yagminas, A., and Foster, W. G. (1999). Effects of acute exposure to PCBs 126 and 153 on anterior pituitary and thyroid hormones and FSH isoforms in adult Sprague Dawley male rats. Toxicol Sci 47, 158-169.

Adult male Sprague-Dawley rats were treated with different doses of either PCB 126 (0, 6.25, 25, 100 or 400 µg/kg) or PCB 153. The PCBs were injected ip daily for two days and serum and thyroid measurements obtained 48 hours later. PCB 126 produced a significant decrease in serum total T_4 at doses of 25 µg/kg and above. Serum total T_3 was significantly decreased in animals given doses of 100 µg/kg and above. Serum TSH was not affected. In a separate experiment 20 mg/kg PCB 153 significantly increased serum T_4 but did not affect serum TSH. Measures of thyroid histopathology were unaffected by either PCB 126 or PCB 153.

The two PCB congeners studied in this report, PCB 126 and PCB 153, differ significantly in their actions on the thyroid system. PCB 126 is a dioxin-like PCB congener and causes a decrease in serum total T_4 without a concomitant effect on serum TSH. In contrast, PCB 153 is a non-coplanar congener with no dioxin-like properties. This PCB congener caused a significant increase in serum T_4. The authors compared this effect with that of estradiol 17-β, concluding that PCB153 may exert an estrogenic effect on serum total T_4. Although this is possible, some PCBs can displace T_4 from the antibody in an RIA, producing a false-positive signal in the RIA (Ruby Bansal, unpublished data). Despite this possible confounding effect, it is clear that different PCB congeners can produce different effects on the thyroid system and that these effects do not conform to an idealized model of HPT responses to reduced circulating levels of thyroid hormone.

C) Meerts, I. A., Assink, Y., Cenijn, P. H., Van Den Berg, J. H., Weijers, B. M., Bergman, A., Koeman, J. H., and Brouwer, A. (2002). Placental transfer of a hydroxylated polychlorinated biphenyl and effects on fetal and maternal thyroid hormone homeostasis in the rat. Toxicol Sci 68, 361-371.

Pregnant Sprague-Dawley rats were treated with 5 mg/kg-day 4-hydroxy-PCB 160 (4OHCB106) by gavage from gestational day 10 to either GD 17 or GD 20. Plasma total and free T_4 and plasma total T_3 was measured in maternal and fetal plasma and in pooled plasma from pups. In dams, 4OHCB106 significantly reduced plasma total T_4 on both GD17 and GD20. However, neither plasma free T_4 nor serum total T_4 was significantly affected. In contrast, plasma total T_4 and plasma free T_4 was significantly reduced in the G20 fetus. Plasma TSH was not affected in the dam, but was significantly increased in the G20 fetus.

These findings demonstrate that a single hydroxylated PCB congener can cause a reduction in circulating levels of T_4, but the effects differ in the dam and pup. Because TSH levels increased only in the pup, it is possible that the HPT axis is responding to this particular toxicant differently at two different life stages.

D) Kodavanti, P. R., Derr-Yellin, E. C., Mundy, W. R., Shafer, T. J., Herr, D. W., Barone, S., Choksi, N. Y., MacPhail, R. C., and Tilson, H. A. (1998). Repeated exposure of adult rats to Aroclor 1254 causes brain region- specific changes in intracellular Ca2+ buffering and protein kinase C activity in the absence of changes in tyrosine hydroxylase. Toxicol Appl Pharmacol 153, 186-198.

Adult Long-Evans rats were dosed daily by gavage with 0, 10, and 30 mg/kg-day Aroclor 1254 for 4 weeks (five times per week). Serum total and free T_4 and T_3 were significantly reduced. Serum TSH was not measured.

E) Kato, Y., Haraguchi, K., Yamazaki, T., Ito, Y., Miyajima, S., Nemoto, K., Koga, N., Kimura, R., and Degawa, M. (2003). Effects of polychlorinated biphenyls, kanechlor-500, on serum thyroid hormone levels in rats and mice. Toxicol Sci 72, 235-241.

Adult male Wistar rats were given a single injection of PCBs (Kanechlor-500) ip. Four days after PCB exposure, blood was taken for analysis of thyroid hormones. Total serum T_4 was significantly elevated by PCB exposure, but T_3 was unaffected. Moreover, serum TSH was unaffected by treatment.

F) Meerts, I. A., Hoving, S., Van Den Berg, J. H., Weijers, B. M., Swarts, H. J., Van Der Beek, E. M., Bergman, A., Koeman, J. H., and Brouwer, A. (2004). Effects of in Utero Exposure to 4-Hydroxy-2,3,3',4',5-Pentachlorobiphenyl (4-OH-CB107) on Developmental Landmarks, Steroid Hormone Levels and Female Estrous Cyclicity in Rats. Toxicol Sci.

Pregnant Sprague-Dawley rats were dosed with 0.5 or 5.0 mg/kg 4-hydroxy-PCB 107 or 25 mg/kg A1254 from GD6 to GD10. Pup serum was taken at postnatal day 4 for analysis of thyroid hormones. Serum total T_4 was significantly reduced by all PCB treatments in both male and female PND4 pups. Serum free T_4 was not affected. Serum total T_3 was affected by 5 mg/kg 4OHPCB107 and A1254 only in males. Serum TSH was not affected.

G) Vansell, N. R., Muppidi, J. R., Habeebu, S. M., and Klaassen, C. D. (2004). Promotion of thyroid tumors in rats by pregnenolone-16alpha-carbonitrile (PCN) and polychlorinated biphenyl (PCB). Toxicol Sci 81, 50-59.

Adult male Sprague-Dawley rats were treated for 20 weeks with PCB 1254 at a dose of 100 ppm in food. Serum hormones were analyzed periodically throughout exposure. Serum total T_4 was significantly reduced by 5 days of treatment and remained consistently reduced throughout the period of exposure. Serum total T_3 was significantly lower than controls periodically and inconsistently and to a much lesser extent than total T_4. Serum TSH was unaffected by treatment.

H) Feder, P. I., and Feng, J. (2005). Draft report on comparison of thyroid activity measures across data sets. EPA Contract No. 68-W-01-023, WA No. 5-3.

Total T_4 was the most sensitive indicator of PCB exposure among these studies. Thyroid weight had the greatest degree of variability.

DISCUSSION

There are a large number of thyroid toxicants identified by their ability to affect circulating levels of thyroid hormones (Brucker-Davis, 1998). According to an idealized model of HPT axis function in which changes in serum T_4 are counterbalanced by the opposite change in serum TSH, one would predict that serum total T_3 would decline secondary to a decrease in serum total T_4, and that serum TSH would become elevated in response. Finally, a common prediction from this model is that thyroid weight and measures of histopathology will respond to changes in serum TSH. These predictions are based on the negative feedback effect of thyroid hormones on TSH and the trophic effects of TSH on the thyroid gland.

The goal of the current analysis was to determine whether this idealized model of HPT axis function was always observed, and to determine which of the endpoints of thyroid function is most sensitive to thyroid toxicants. For example, while it is predicted that thyroid weight and histology would change in response to changes in serum TSH, it is possible that the technical features of the TSH assay make it more difficult to detect a change than are changes in thyroid weight. Moreover, because thyroid hormones and TSH exhibit various rhythms in the blood, it is possible that single point estimates of hormone levels are quite variable and that thyroid weight/histology represents a sensitive measure of changes in TSH integrated over the duration of toxicant exposure.

A surprisingly small number of studies were identified that reported all of our target measurements (T_4, T_3, TSH, thyroid weight and histology). However, this was sufficient for a quantitative analysis performed by Feder and Feng (Feder and Feng, 2005) for perchlorate and for PTU. In contrast, we identified very few studies of PCBs in which all of these measures were obtained. Moreover, we did not identify studies on PCBs in which the characteristics of the experiments were similar.

Our qualitative analysis of these studies supports several conclusions. First, there are genuine differences in the way the HPT axis responds to chemicals acting on the system by different modes of action. Propylthiouracil blocks the TPO enzyme (Nagasaka and Hidaka, 1976; Sugawara, 1985) and may well be the principal compound used to define the hormonal relationships among levels of the HPT axis. Considering this, it may not be surprising that PTU produced effects on the HPT axis that are most consistent with an idealized model. Serum total T_4 was always reduced by PTU and the dose at which this occurred coincided generally with a decrease in serum total T_3, and increase in TSH and an increase in thyroid weight. Minor differences between studies are not deemed to be important, other than to say that a NOEL for PTU differs slightly between studies. The quantitative analysis by Feder and Feng suggests that changes in thyroid histopathology are likely to be the most sensitive indicator of PTU effects. This

observation is consistent with the interpretation that relatively subtle histological characteristics of the thyroid gland, not associated with changes in thyroid weight, represent an integrated measure of small changes in serum TSH that are not detectable by RIA.

Despite these observations with PTU exposure, treatment with perchlorate or with PCBs did not replicate this pattern. Serum total T_3 appeared to be the most sensitive measure to perchlorate exposure in the qualitative analysis, though the analysis by Feder and Feng indicated that measures of thyroid histology were relatively more sensitive. More than in the case of PTU, there were greater differences in the effects of perchlorate on the HPT axis among studies. For example, York *et al.* (2001) found an "inverted U" dose response with perchlorate on serum total T_4. Thus, serum total T_4 is increased by low doses of perchlorate and reduced below control levels by higher doses of perchlorate. Although this study was only one showing this response curve, several reports in the literature, from human and animal studies, have reported similar findings (citations?).

The HPT response to PCB exposure was subtly different from that of both PTU and of perchlorate. Specifically although serum total T_4 was decreased by PCB exposure in each case, serum TSH was not often reported to be changed. This appears to be characteristic of PCB exposure in that there are a number of similar reports even in this small sub-sample. Thyroid weight was relatively sensitive to PCB exposure.

In conclusion, inclusion of a comprehensive battery of serum total T_4, T_3, TSH and thyroid weight and histopathology in screens and tests for thyroid toxicants is indicated by a review of three known thyroid toxicants acting through different mechanisms. However, the age and duration of exposure and animal gender are likely to impact the sensitivity of the HPT axis to some or all toxicants. Moreover, it appears unwarranted to postulate that the HPT axis will respond similarly to all known toxicants, as the literature reviewed here for three toxicants does not support this conclusion. Perchlorate exhibited the most variable effects among reviewed studies and PTU was the least variable. This may be due to variables other than the toxicant (e.g., age, duration of exposure, etc) and the relatively small number of studies reviewed.

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APPENDIX A.1

Pool of Reports on Perchlorate Effects on the HPT Axis

The following references were identified in PubMed using the key words "perchlorate," "thyroid weight," and "rat."

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APPENDIX A.2

Pool of Reports on PTU Effects on the HPT Axis

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APPENDIX A.3

Pool of Reports on Polychlorinated Biphenyl Effects on the HPT Axis

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APPENDIX B

DRAFT REPORT ON COMPARISON OF THYROID ACTIVITY MEASURES ACROSS DATA SETS (FEDER AND FENG 2005)

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Draft Report

on

COMPARISON OF THYROID ACTIVITY MEASURES ACROSS DATA SETS

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EXECUTIVE SUMMARY

The relative sensitivities of four continuous activity measures (TT3, TT4, TSH, and thyroid weight) and one ordinal response measure activity measure were compared based on data from multiple dose response rat feeding studies reported in the literature, with chemicals perchlorate, PTU, and PCB. The test procedures varied with respect to numbers and levels of does, dose durations, and animal age and gender.

Sensitivity of an activity measure was expressed in terms of steepness of the dose response, as indicated by the slope of the dose response relation. The activity measures were ranked based on the lower bounds of the 95 percent confidence intervals on absolute slope.

For perchlorate the analysis indicated that hypertrophy was relatively sensitive compared to the continuous measures and that TT3 and TT4 were relatively less sensitive than TSH and thyroid weight. The relatively high sensitivity of hypertrophy was heavily influenced by the results from a single study, York 2001.

For PTU and PCB the inference sensitivity for comparisons among measures was lower than for perchlorate because there was less data available. For PTU there was no indication of sensitivity differences among measures. For PCB there was no hypertrophy data. TT4 had relatively higher sensitivity than the other continuous measures.

A sensitivity analysis was carried out in which an alternative method was used to calculate the slopes of the continuous activity measures. This was done to determine whether the relatively high average sensitivity observed for hypertrophy in the perchlorate studies would still hold. For perchlorate the hypertrophy average sensitivity was still relatively high compared to the average sensitivities of the continuous measures. This again was in large part due to the sensitivity ranks in the York 2001 study. The relative sensitivities of the four continuous activity measures differed somewhat from that determined with the logarithmic analysis. They were all equivalent.

For PTU the relative sensitivities agreed with those determined with the original analysis. The confidence bands were wider than those for perchlorate and overlapped across all the measures. For PCB the confidence band for thyroid weight was higher than those for the other measures.

In summary the relation between the sensitivities of the continuous response measures and that of the ordinal response measure agreed between both sets of calculations. The relations among the continuous measures differed between the two sets of calculations. Since the second set of calculations assumed constant standard deviation across doses, which was sometimes violated, the relations among the continuous measures based on the first analysis may be more appropriate.

1.0 INTRODUCTION AND BACKGROUND

Dr. Thomas Zoeller is conducting a literature review under Work Assignment 5-5, Task 13 to compare the sensitivities of alternative measures of thyroid activity. He has identified multiple *in vivo* studies on rats in which thyroid activity was measured at graded doses of toxicant. The measures considered by Dr. Zoeller were TT3, TT4, TSH, thyroid weight, and histopathological evaluations of hypertrophy/hyperplasia. The toxicants considered by Dr. Zoeller were perchlorate, PTU, and PCB. The TT3, TT4, TSH, and thyroid weight measures are continuous responses. The hypertrophy/hyperplasia measure is an ordered categorical response.

The various studies differ with respect to animal characteristics (stage of life, age, gender), dose levels, dose units, dose duration. The individual studies are divided into "data segments" corresponding to differing animal ages, genders, and dose durations. It is not *a priori* clear which "data segments" are comparable and can be combined to determine overall estimates of relative sensitivity. Also it is not clear whether the PCB congeners are consistent across the PCB studies.

Combining raw data across studies and data segments could be problematic. Subsets of studies and subsets of "data segments" within studies would need to be selected that are comparable on biological grounds. This would require selecting which data portions to include or not include in the analyses and could lead to criticism about the data choices.

Although the test conditions can differ considerably across "data segments", they are consistent across activity measures within "data segments". An alternative approach to combining data across studies is to determine measures of endpoint sensitivity for each endpoint within each "data segment" and then determine measures of relative sensitivity across endpoints within each segment. Measures of relative sensitivity across endpoints within "data segments" account for differences in study conditions across data segments. The measures of relative sensitivity within "data segments" can then be compared across data segments to determine if any of the measures are consistently more sensitive. Such an approach was adopted for the analysis discussed in this report.

The remainder of the report is organized as follows. Section 2 includes a brief outline of the data sources used. Section 3 presents the statistical methods used. Section 4 presents the analysis results. Section 5 contains a summary and discussion of results. Appendices I, II, and III display plots of the trends in thyroid activity response versus increasing dose.

2.0 DATA SOURCES

The statistical analyses in this report are based on multiple dose response thyroid activity studies with rats. The data were compiled by Dr. Thomas Zoeller as part of his literature review for Work Assignment 5-5, Task 13. The studies involve graded doses of perchlorate, PTU, or PCB at various dose levels, dose durations, and animal life stages. The number of perchlorate "data segments" was substantially greater than the number of PTU "data segments" or the number of PCB "data segments".

The following studies and "data segments" were used in the analyses. See Dr. Zoeller's discussion for information concerning these studies.

1. Perchlorate
   - a. Hiasa
     - i. Adult male
   - b. Mahle
     - i. Adult female
     - ii. Pup male
     - iii. Pup female
     - iv. Fetuses
   - c. Siglin
     - i. Adult male-14 days, 90 days, 30 day recovery
     - ii. Adult female-14 days, 90 days, 30 day recovery
   - d. York 2001
     - i. Adult male
     - ii. Adult female
     - iii. Pup male

iv. Pup female
e. York 2002
i. Fetus
f. York 2003
i. Pups

2. PTU
a. Cho
i. Adult male
ii. Adult female
b. Hood
i. Adult male
c. Mellert
i. Adult male
ii. Adult female
d. O'Connor
i. Adult male

3. PCB
a. Bowers
i. Adult female
ii. Pups
b. Desaulniers
i. Adult male
c. Kato
i. Adult male
d. Kodavanti
i. Adult male
e. Meerts 2002
i. Pup-GD 17, 20
f. Meerts 2004
i. Adult male
ii. Adult female
g. Vansell
i. Adult male

3.0 STATISTICAL METHODS

Assume that within each "data segment" each sensitivity measure has a monotone relation with dose (either increasing or decreasing). The validity of this assumption is displayed for the measured results in the plots in Appendices I, II, or III.

For the continuous measures (TT3, TT4, TSH, thyroid weight) let

$Y \equiv$ response
$X \equiv \log(1+\text{Dose})$

Approximate the monotone relation by a straight line relation between the logarithm of measure Y and logarithm of dose X (natural logarithms are used). Namely

$$\log(Y) = \alpha + \beta\log(1+D) + \varepsilon \equiv \alpha + \beta X + \varepsilon$$

(Adding 1 to the dose makes the control group correspond to 0 on the log scale and the other dose groups correspond to positive values on the log dose scale.) The expected logarithmic activity in the control group ($\log(1+D) = \log(1) = 0$) is $E[\log(Y_0)] = \alpha$. The expected logarithmic activity at (logarithm of dose) X is $E[\log(Y_D)] = \alpha + \beta X$. This can be represented as relative change in activity level compared to the control group.

$$E[\log(Y_D/Y_0)] = \beta X$$

The log scale adjusts for different units of measurement and different sized values among the various measures by representing relative change.

For the ordinal histopathology data at (logarithm of dose) X

$$P(\text{response is in Category 1}) = \Phi(\alpha + \beta X)$$
where $\Phi(.)$ is the standard normal cdf

$$P(\text{response is in Category i or lower}) = \Phi(\alpha + C_i + \beta X)$$
$i=2,...,K-1$

$$P(\text{response is in Category K}) = 1 - \Phi(\alpha + C_{K-1} + \beta X)$$

The conceptual physical interpretation of the ordinal model is that there is an underlying continuous variable, Z, that measures extent of hypertrophy. Z is normally distributed with mean $\mu_X \equiv \gamma_0 + \gamma_1 X$ and standard deviation σ. The exact values of Z cannot be observed, only categorized values, given labels such as "none", "mild", "moderate", "marked". The boundaries of these categories are distribution cut points in the Z scale at unknown values $\xi_1, \xi_2,...,\xi_{K-1}$. The probability of being less than or equal to ξ_i (i.e. in Category i or lower) is $\Phi\left(\frac{\xi - \mu_X}{\sigma}\right)$.

Comparison with the above expressions implies that

$$\alpha + C_i + \beta X = \frac{\xi - \mu_X}{\sigma} \equiv \frac{\xi - (\gamma_0 + \gamma_1 X)}{\sigma}$$

This implies that the estimated slope β in the ordinal response model is related to the slope γ_1 of the conceptual underlying continuous variable, Z, by $\beta = (-\gamma_1/\sigma)$. Thus the slope β fitted in the ordinal response model is interpreted as the negative of the slope of underlying conceptual measured variable normalized to unit standard deviation.

It was suggested by Dr. Zoeller that changes in response with increasing dose indicate toxicity irrespective of whether the changes are positive or negative. The steeper the slope the greater is the toxic effect. Thus the magnitudes of the fitted slopes are compared across activity measures, and not their signs. If the fitted slope is positive the slope value is used for comparison. If the fitted slope is negative the negative of the slope is used for comparison. Thus in the discussion below it is assumed that the slope is positive.

Let R (e.g. R=1.2) denote a specified amount of relative change (i.e. $Y_D = RY_0$). The (logarithmic) dose X that corresponds to an increment $\log(Y_D/Y_0) = r \equiv \log®)$) (e.g. $r = \log(1.2) = 0.22$) is

$$X = r/\beta$$

The parameters α, β are estimated for each activity measure within each "data segment" based on weighted linear regression model fits. The weights are the reciprocals of the squares of the standard errors of the observations. Let $\hat{\beta}, \beta_L$ denote the regression estimate of β and the lower 95% confidence bound respectively. We wish to determine a lower bound, X_r, on X such that it can be inferred that if $X > X_r$ then with probability 0.95, $\beta X > r$ The steeper the slope β, the smaller will X be.

By definition of the lower 95 % confidence bound, β_L, $P[\beta > \beta_L] = 0.95$. Thus $P[\beta X > \beta_L X] = 0.95$. Assume $\beta_L > 0$. If we choose X_r such that $\beta_L X_r = r$ (i.e. $X_r = r/\beta_L$) then we can be 95% confident that

$$\text{If } X > X_r \equiv r/\beta_L \text{ then } \beta X > \beta X_r > \beta_L X_r = r$$

Thus assuming that $\beta_L > 0$ the relative sensitivity of activity measures within each data segment can be compared based on the values of r/β_L. The endpoint with the smallest value of r/β_L is the most sensitive. Equivalently the endpoint with the largest value of β_L is the most sensitive.

Rank the sensitivities of the activity measures in order of their values of β_L. Extend this ranking to those measures with $\beta_L < 0$. This then provides a basis for ranking all the activity measures within a "data segment". The activity measure with the largest value of β_L is the most sensitive.

Note that the above ranking procedure is applied to the ordinal histopathology activity measure as well as to the continuous activity measures. For the continuous measures the slope corresponds to relative change in mean value of response. For the ordinal measure the slope corresponds to the negative of slope of the underlying measurement normalized to standard deviation 1. We will compare the ordinal histopathology activity measure with the unnormalized continuous measures, but caution should be exercised in interpreting their relation.

The following ranking procedure was adopted within each "data segment". For each of the four continuous activity measures that were included in the "data segment" a linear regression model was fitted relating the (natural) logarithm of activity to log(1+Dose) by weighted least squares, using the reciprocals of the squares of the standard errors as weights. This resulted in slope estimates and 95% lower confidence bounds $\beta_{L.}$ If the "data segment" included ordinal histopathology data an ordinal data regression model was fitted to the data relating the probability of being in each response category to log(1+Dose) by maximum likelihood analysis.

The model fitting procedures resulted in a maximum of five estimated slopes $\hat{\beta}$ and associated lower confidence bounds β_L within data segment. The $\beta_{L.}$'s for each measure were ranked from smallest to largest (i.e. rank 1 corresponding to the smallest,..., rank 5 corresponding to the largest). Thus if the "data segment" included all five activity measures, the measures would have a set of ranks 1,2, 3, 4, 5. The average rank is 3. If there were fewer than five (e.g., three) activity measures the ranks in the "data segment" would be 1, 2, 3. For purposes of comparisons across "data segments" the ranks were scaled so that the average rank remained 3. For example with three activity measures, the scaled ranks assigned would be 1.5, 3, 4.5; with four activity measures, the scaled ranks assigned would be 1.2, 2.4, 3.6, 4.8. If an activity measure is more sensitive than the others it would be expected to have ranks mostly 4 or 5 in each "data segment". If all the activity measures were equally sensitive the ranks for each measure would be expected to be uniformly distributed across 1, 2, 3, 4, 5. Note that this analysis treats the "data segments" within each study as independent segments.

A preliminary analysis of variance was carried out on the ranks pooled across the three chemicals. The fixed effect factors in the analysis of variance were

- Chemical (perchlorate, PTU, PCB)
- Animal category (male, female, pup)
- Activity measure (TT3, TT4, TSH, thyroid weight, histopathology)

and their interactions. If Chemical or Animal category and their interactions with Activity measure are nonsignificant then comparisons among activity measures can be pooled across Animal category or Chemical.

The interpretation of the slope β estimated by the ordinal response method is $\beta = (-\gamma_1/\sigma)$ where γ_1 is the dose response slope for the "conceptual continuous measure" underlying the ordinal response categories and σ is the standard deviation of the "conceptual continuous measure". Thus β is the slope normalized for standard deviation. For the continuous measures the slopes are based on logarithmic transformations of the responses and are not normalized for logarithmic standard deviation.

A sensitivity analysis was carried out to determine if the results were sensitive to the method of analysis used to estimate the slopes of the continuous activity measures. An alternative method was used to estimate slopes and associated standard errors. The method was motivated by the interpretation of the ordinal slopes. It was assumed that within each "data segment" the standard deviation of the activity measure is approximately constant across doses (but may vary among activity measures). The data were normalized by their standard deviations at each dose. This corresponds to basing analyses on the reciprocals of the coefficients of variation. The normalized values are unitless. A linear regression was fitted to the normalized responses for each activity measure within each data segment by weighted least squares regression analysis, with weights being the sample sizes associated with the doses. This resulted in estimated slopes and lower 95 percent confidence bounds for each of the continuous activity measures within each "data segment". The same ordinal analysis slopes as discussed above were used for hypertrophy.

The $\beta_{L.}$'s for each measure were ranked from smallest to largest (i.e. rank 1 corresponds to the smallest,..., rank 5 corresponds to the largest). Thus if the "data segment" included all five activity measures, the measures would have a set of ranks 1,2, 3, 4, 5. The average rank is 3. Analysis of variance was carried out on the ranks. Least squares means and associated 95 percent confidence intervals were calculated for each activity measure and compared across measures. The results of these analyses are displayed in the results section.

4.0 RESULTS

A. Monotonicity

The assumption was made that the continuous activity measures have monotonic relations with dose (either increasing or decreasing). To assess the validity of this assumption the dose response relations for each continuous activity measure within each "data segment" were plotted. The plots relate log(activity measure) to log(1+Dose). The plots are displayed in Appendices I, II, and III, corresponding to perchlorate, PTU, and PCB respectively. These plots indicate that in nearly all cases the monotonicity assumption is valid.

B. Regression Analysis

Regression analyses were carried out within each "data segment" to relate measured response to dose. For the continuous activity measures the mean, standard deviation, and sample size were available for each dose group. This permitted the standard error to be calculated.

For each of the continuous activity measures weighted linear regression models were fitted to describe the relation between log(activity measure) and log(1+Dose). For the ordinal histopathology activity measure a linear relation was fitted to describe the relation between probability of being in each response category and log(1+Dose). The estimated slopes and associated standard errors for each activity measure within each study and data segment are displayed in Tables 1, 2, and 3, for perchlorate, PTU, and PCB respectively. Several of the histopathology slope estimates have extremely large standard errors (in the thousands or greater): in particular Siglin-perchlorate, 14 day and 90 day females; Cho-28 days male-PTU; Mellert-PTU males; O'Connor-PTU. The slopes for the hypertrophy measure are for the most part the largest in absolute value, sometimes by one or more orders of magnitude.

C. Ranks

Following the discussion in the methods section the activity measures were ranked based on the lower 95 confidence bounds on the absolute values of the slopes. A rank of 1 corresponds to the smallest (i.e. least positive or most negative) lower bound, ..., a rank of 5 corresponds to the largest lower bound. If one or more of the activity measures was not determined for that "data segment" the ranks range from 1 to the number of activity measures determined. The ranks for each activity measure within each study and data segment are displayed in Tables 4, 5, and 6, for perchlorate, PTU, and PCB respectively. In the five cases mentioned above where the standard error for the hypertrophy measure was very large the assigned rank was 1. In these cases the slopes may indeed be large (in absolute value) however the data may not be sufficient to estimate them, as indicated by the standard errors in the thousands or greater. Other than the cases where the hypertrophy measure was effectively nonestimable, the hypertrophy measure mostly had the highest rank.

D. Analysis of Variance

Analysis of variance was carried out on the ranks in Tables 4, 5, 6, combined. "Data segment" (group_id) was included as a random effect. Animal category (male, female, pups), chemical (perchlorate, PTU, PCB), and activity measure (TT3, TT4, TSH, thyroid weight, hypertrophy) were included as fixed effects. For those "data segments" with less than five activity measures the ranks were scaled so that the average rank was 3. For example for perchlorate-Mahle-GD 20-dams there is data on just three activity measures - TT3, TT4, TSH. The ranks 1, 2, 3 displayed in Table 4 were scaled up to 1.5, 3, 4.5 for purposes of the analysis of variance. The scaling was intended to reduce or eliminate a downward bias in average rank for those activity measures that were included in the "data segments" with fewer than five activity measures available.

The results of the analysis of variance are displayed in Table 7. The "data segment" random effect has 0 variation, as would be expected since the average ranks within each segment were maintained constant at 3. Chemical by activity measure interaction is highly significant. Animal category by activity measure interaction is not significant. This implies that comparisons among the activity measures may be pooled across animal categories but should not be pooled across chemicals.

Separate analyses of variance were carried for each chemical and animal category with factor activity measure. Least squares means and associated 95 percent confidence intervals for activity measure were determined and are plotted in Figures 1, 2, and 3 for perchlorate, PTU, and PCB respectively. The horizontal reference lines in each figure correspond to the average rank across activity measures within each data segment. The widths of the confidence bands are related to the number of "data segments" for that animal category and activity measure as well as the residual variability. For example for PCB and TSH the data included 1 adult female segment, 3 adult male segments, and 3 pup segments. The confidence band for females is wider than those for males or pups.

For the most part for each activity measure the average rank is similar across animal categories. The confidence bands overlap across activity measures so there is no evidence of statistically significant differences among activity measures. However for perchlorate the average ranks for TT3 and to a lesser extent TT4 are a little lower than those for TSH and thyroid weight, which in turn are a little lower than those for hypertrophy. For PTU and PCB the confidence bands are wider and the average ranks are similar across activity measures.

Since the animal category main effect and the activity measure by animal category interaction were not significant in the analysis of variance, separate analyses of variance were carried for each chemical, with factors animal category and activity measure. Least squares means and associated 95 percent confidence intervals for activity measure averaged across animal category were determined and are plotted in Figures 4, 5, and 6 for perchlorate, PTU, and PCB respectively. The horizontal reference lines in each figure correspond to the average rank across activity measures within each data segment. For perchlorate the average rank for TT3 is significantly lower than that for TT4, which in turn is lower than those for TSH and thyroid weight. These are a little lower than that for hypertrophy. For PTU the confidence bands are wider than those for perchlorate. The activity measures have similar average ranks with TSH a little bit higher. Since the confidence bands overlap considerably there is no statistical evidence of differences among activity measures. For PCB the average ranks for TT3, TSH, and thyroid weight are similar. The average rank for TT4 is higher. There is no hypertrophy data for PCB. In summary, the comparisons among activity measures are not consistent across chemicals. For perchlorate TT4 is relatively low whereas for PCB it is relatively high. For perchlorate hypertrophy is relatively high whereas for PTU it is in line with the other measures. The relatively high average rank for hypertrophy in the perchlorate results is heavily dependent on the York 2001 study results, where it is consistently the most sensitive across all

the animal categories. For perchlorate, TSH and thyroid weight are higher than TT4 whereas for PCB they are lower.

It was discussed above that the slope associated with hypertrophy, which is based on ordinal pathology measures, may not be directly comparable to the slopes associated with TT3, TT4, TSH, and thyroid weight, which are based on continuous measures. The hypertrophy measure was omitted from the analysis and the four continuous measures were compared among one another. For perchlorate the hypertrophy measure had rank 1 or 5 in every "data segment" in which pathology data occurred (Table 4). For PTU the hypertrophy measure had rank 1 or the highest rank (4 or 5) in every "data segment" in which pathology data occurred (Table 5). For PCB there was no hypertrophy data. This suggests that among the four continuous activity measures the relative sizes of the ranks, and therefore the average ranks, would be nearly the same whether hypertrophy was included or excluded. Analysis of variance was carried out for the four continuous activity measures after excluding hypertrophy. Displays analogous to Figures 1 and 2 were prepared for perchlorate and PTU based on the least squares means of the ranks after excluding hypertrophy. These are displayed in Figures 7 and 8 respectively. Figure 7 is similar to Figure 1 and Figure 8 is similar to Figure 2. Thus the relative sensitivities among the continuous activity measures do not depend on whether or not hypertrophy is included.

E. Sensitivity Analysis

Weighted least squares regression analyses were carried out on the activity measures normalized by their standard deviations (with no log transformation) and the slopes, standard errors, and 95 percent confidence intervals were determined. The lower confidence bounds were ranked from ranked from smallest to largest (rank 1 corresponding to the smallest,..., rank 5 corresponding to the largest). The ranks for perchlorate, PTU, PCB are displayed in Tables 8, 9, and 10 respectively. Comparison of Tables 4, 5, 6 (based on the logarithmic fits) with Tables 8, 9, 10 (based on the standardized data fits for the sensitivity analysis) shows that the hypertrophy measure has nearly the same ranks in both analyses. It is either the most sensitive or the least sensitive depending on whether the slope can be estimated. It is particularly sensitive in the York 2001 study with perchlorate. The ranks for the continuous activity measures differ between the two sets of tables.

Analysis of variance on the ranks calculated in the sensitivity analysis based on normalized data indicated that animal characteristic main effect and measure by animal characteristic interaction were not significant but that measure by chemical interaction was significant. Thus the least squares means for the ranks are reported combined across animal characteristics but separately for each chemical. The least squares means and associated 95 percent confidence intervals are displayed in Figures 9, 10, and 11 for perchlorate, PTU, and PCB respectively.

Figure 9 shows that for perchlorate the hypertrophy measure has a larger average rank than do the other measures, although the confidence intervals overlap so the differences are not statistically significant. The other four activity measures are statistically equivalent and in contrast to Figure 4 the average ranks for TT3 and TT4 are comparable to those of TSH and thyroid weight. It should be noted that the relatively high average rank for hypertrophy is due in large part to the York 2001 study in which hypertrophy is consistently more sensitive than the other activity measures.

Figure 10 shows that for PTU the activity measures are equivalent. This is in agreement with Figure 5. It should be noted that the average performance of hypertrophy for PTU is an average of relatively sensitive performance for two female studies and relatively insensitive performance for three male studies.

Figure 11 differs from Figure 6. Figure 11 shows relatively high average rank for thyroid weight and equivalent average rank for the other three measures. Figure 6 shows relatively high average rank for TT4 and equivalent average rank for the other three measures. It should be noted that the relatively high average rank of thyroid weight is based on just two "data segments", corresponding to the Kato and Vansell studies. Each data segment includes just a control dose and a single test dose (Vansell-0 and 100 ppm with n=24 per group; Kato-0 and 100 mg/kg-day with n=5 per group).

5.0 SUMMARY AND DISCUSSION

The relative sensitivities of four continuous activity measures (TT3, TT4, TSH, and thyroid weight) and one ordinal response measure (e.g. none, mild, moderate, severe response categories) activity measure were compared based on data from multiple dose response rat feeding studies reported in the literature, with chemicals perchlorate, PTU, and PCB. The test procedures varied with respect to numbers and levels of dose, dose durations, and animal age and gender. Each study was divided into "data segments" corresponding to homogeneous subsets, e.g. adult males, pups, 21 day dose duration, etc. The "data segments" were broadly divided into three groupings based on animal characteristics: adult males, adult female, pup/fetus. For analysis purposes each "data segment" was treated as an independent entity.

Sensitivity of an activity measure was expressed in terms of steepness of the dose response, as indicated by the slope of the dose response relation. It was not of concern whether the dose response was increasing or decreasing. A rationale was made for ranking the measures based on the lower bound of the 95 percent confidence interval on absolute slope.

For the four continuous measures dose response slopes were determined based on dose response relations between the logarithm of response and the logarithm of dose. For the ordinal response the dose response slope was based on an ordinal response model fit to the category probabilities.

For perchlorate the analysis indicates that hypertrophy is relatively sensitive compared to the continuous measures and that TT3 and TT4 are relatively less sensitive than TSH and thyroid weight. It should be noted that the relatively high sensitivity of hypertrophy is heavily influenced by the results from a single study, York 2001 (eight of 14 "data segments"). This study had eight "data segments", within each one hypertrophy was the most sensitive. In the other studies that included hypertrophy (York 2003, York 2004, Siglin) hypertrophy was the most sensitive when the slope could be estimated (half the cases) and the least sensitive when the slope could not be estimated.

For PTU and PCB the inference sensitivity for comparisons among measures was lower than for perchlorate, as indicated by the greater widths of the confidence bands around the average ranks. This is because the studies for these chemicals had fewer "data segments" as compared to perchlorate. For PTU there was no indication of sensitivity differences among measures, as indicated by the overlap among the confidence bands. For PCB there was no hypertrophy data. TT4 had relatively higher sensitivity than the other continuous measures.

A sensitivity analysis was carried out in which an alternative method was used to calculate the slopes of the continuous activity measures. This was done to determine whether the relatively high average sensitivity observed for hypertrophy in the perchlorate studies would still hold. The method for calculating the slopes of the continuous measures was selected to mimic the physical interpretation of the hypertrophy slope, as discussed in the methods section. For perchlorate the hypertrophy average sensitivity was still relatively high compared to the average sensitivities of the continuous measures. This again was in large part due to the sensitivity ranks in the York 2001 study. The relative sensitivities of the four continuous measures differed somewhat from that determined with the logarithmic analysis. They were all equivalent.

For PTU the relative sensitivities agreed with those determined with the logarithmic analysis. The confidence bands were wider than those for perchlorate and overlapped across all the measures.

For PCB the confidence band for thyroid weight was higher than those for the other measures. This differed from the results of the logarithmic analysis, where thyroid weight was a little low. These results need be interpreted with caution since the thyroid weight sensitivity determination was based on just two studies, Kato and Vansell. Each study included a control group just one nonzero dose group. The Kato study included just five animals per dose group. An important assumption underlying the estimation procedure used in the sensitivity analysis was of constant standard deviation across dose groups. This assumption is clearly violated in the Vansell study, where the standard deviation was 4.33 in the control group and 37.09 in the high dose group. It was also violated, but to a lesser extent, in the Kato study, where the standard deviations were 0.00213 in the control group and 0.00064 in the high dose group. Since the standard deviations were used to standardize the mean values before determining slopes, departures from constant standard deviation could clearly distort the slope estimates, particularly with just two dose groups. Thus the logarithmic analysis results appear to be more reasonable.

In summary the relation between the slopes of the continuous response measures and that of the ordinal response measure agreed between both sets of calculations. The relations among the continuous measures differed between the two sets of calculations. Since the second set of calculations assumed constant standard deviation across doses, which was sometimes violated, the relations among the continuous measures based on the logarithmic analysis may be more appropriate.

Several data considerations may impact the results somewhat. As discussed above, the sensitivity analysis method for determining the slopes of the continuous activity measures assumed that that the standard deviations within “data segments” were constant across doses. Means were normalized by the observed standard deviations. This assumption held reasonably well for many of the “data segments” however in some “data segments” the standard deviations varied by factors of two or three across doses. This could distort the slope estimates. The first set of comparisons among the continuous measures might thus be given greater weight than the second.

There were difficulties interpreting several of the standard deviations reported in the data sets. For the York 2001 perchlorate study the reported standard deviation for TT4 for P-adult females, dose 0.3 mg/kg-day was 0.04. This was 20 to 25 times lower than any of the other TT4 standard deviations reported. This standard deviation was checked and was reported as being correct so it was used in the analyses. Several of the standard deviations were reported as 0. For example O’Connor, Hood, Cho - PTU. In these situations standard deviations values were imputed based on surrounding values in order to calculate weights. The standard deviations were assumed to be approximately proportional to mean value.

The confidence bands for PCB females in Figure 3 are wide, particularly for TSH. This is because there were just two female PCB studies. When females were considered by themselves there was just one degree of freedom to estimate error. For TSH there was just one female study, so the confidence band was particularly wide. In general the amount of data for PTU and PCB studies was less than that for perchlorate studies. Therefore confidence bands were wider and inference sensitivity was lower.

Table 1. Slope and Associated Standard Error for Each Activity Measure Within Each Data Segment. Perchlorate

Perchlorate								
Study	**Group**	**Gender**	**Category**	**TT3**	**TT4**	**TSH**	**TW**	**HT**
Hiasa	350g male	Male	Male	-0.001 (0.245)	-0.038 (0.232)	0.107 (0.235)	0.096 (0.207)	
Mahle	GD 20 dam	FEMALES	Female	-0.140 (2.806)	-0.730 (2.159)	0.742 (2.059)		
Mahle	GD 20 fet	NA	Pup	-0.130 (2.075)	-0.206 (2.055)	0.387 (2.055)		
Mahle	PND 10 da	Females	Female	-0.067 (2.751)	-0.136 (2.124)	0.250 (2.058)		
Mahle	PND 10 pu	Females	Pup	-0.042 (2.643)	-0.370 (2.142)	0.584 (2.057)		
Mahle	PND 10 pu	Male	Pup	-0.062 (2.659)	-0.125 (2.164)	0.189 (2.056)		
Siglin	14 days	FEMALES	Female	-0.022 (0.658)	-0.069 (0.505)	0.148 (0.483)	-0.000 (0.476)	-4.411 (22860.11)
Siglin	14 days	Male	Male	-0.106 (0.764)	-0.093 (0.519)	0.163 (0.488)	0.033 (0.476)	-1.686 (0.350)
Siglin	30 day re	FEMALES	Female	-0.050 (0.977)	-0.045 (0.542)	0.047 (0.520)	0.014 (0.515)	
Siglin	30 day re	Male	Male	-0.051 (0.927)	-0.108 (0.549)	0.032 (0.529)	0.000 (0.515)	
Siglin	90 days	FEMALES	Female	-0.081 (0.687)	-0.088 (0.496)	0.074 (0.481)	0.033 (0.476)	-4.852 (22471.15)
Siglin	90 days	Male	Male	-0.088 (0.725)	-0.157 (0.511)	0.047 (0.487)	0.031 (0.476)	-1.178 (0.237)
York 2001	F1 adult	Females	Female	0.003 (0.611)	0.001 (0.439)	0.111 (0.382)	0.023 (0.369)	-0.909 (0.116)
York 2001	F1 adult	Males	Male	0.004 (0.458)	-0.102 (0.405)	0.226 (0.381)	0.049 (0.369)	-0.751 (0.096)
York 2001	F1 pup	Females	Pup	-0.029 (0.458)	-0.043 (0.441)	0.033 (0.386)	0.004 (0.369)	-1.130 (0.137)
York 2001	F1 pup	Males	Pup	0.000 (0.502)	0.002 (0.449)	0.020 (0.411)	0.005 (0.369)	-0.903 (0.116)
York 2001	F2 pup	Females	Pup	-0.025 (0.620)	0.040 (0.421)	0.008 (0.586)	0.003 (0.369)	-1.184 (0.136)
York 2001	F2 pup	Males	Pup	0.004 (0.582)	0.016 (0.426)	0.039 (0.474)	0.001 (0.369)	-0.993 (0.168)
York 2001	P adult	Females	Female	-0.001 (0.673)	-0.003 (0.410)	0.006 (0.376)	0.013 (0.369)	-1.327 (0.160)
York 2001	P adult	Males	Male	0.005 (0.480)	-0.077 (0.411)	0.276 (0.402)	0.023 (0.369)	-1.446 (0.171)
York 2003	DG 21 fet	NA	Pup	-0.068 (0.361)	-0.028 (0.343)	0.108 (0.341)	0.092 (0.353)	0.042 (0.388)

Perchlorate								
Study	**Group**	**Gender**	**Category**	**TT3**	**TT4**	**TSH**	**TW**	**HT**
York 2004	F1 PUPS	NA	Pup	-0.394 (0.565)	-0.129 (0.520)	0.077 (0.503)	0.006 (0.501)	-0.157 (0.167)

Table 2. Slope and Associated Standard Error for Each Activity Measure Within Each Data Segment. PTU

Study	**Group**	**Gender**	**Category**	**TT3**	**TT4**	**TSH**	**TW**	**HT**
Cho	28days	females	Female	-0.090 (0.543)	-0.435 (1.457)	0.078 (0.520)		-2.486 (0.983)
Cho	28days	males	Male	-0.146 (0.615)	-1.397 (1.255)	0.007 (0.520)		-7.758 (11.790)
HOOD	21 days	NA	Male	-0.203 (0.201)	-0.490 (0.294)	0.345 (0.276)	0.250 (0.469)	
Mellert		Females	Female	-0.439 (0.533)	-0.576 (0.556)	0.996 (0.661)	0.854 (0.664)	-1.858 (0.418)
Mellert		Males	Male	-0.505 (0.567)	-0.760 (0.551)	0.893 (0.613)	0.867 (0.631)	-4.491 (4.6505E8)
O'Connor	Group One	NA	Male	-0.348 (0.360)	-2.150 (0.361)	0.375 (0.366)	0.029 (0.359)	-177.752 (1.2878E8)

Table 3. Slope and Associated Standard Error for Each Activity Measure Within Each Data Segment. PCB

PCB							
Study	**Group**	**Gender**	**Category**	**TT3**	**TT4**	**TSH**	**TW**
Bowers	dams	female	Female	-0.075 (0.731)	-0.148 (0.513)	0.088 (0.552)	
Bowers	offspring	NA	Pup	-0.112 (0.612)	-0.217 (0.516)	0.049 (0.541)	
Desaulniers		male	Male	-0.099 (0.265)	-0.166 (0.226)	0.029 (0.230)	
Kato		NA	Male	-0.017 (0.307)	-0.349 (0.308)	-0.063 (0.365)	0.000 (0.306)
Kodavanti		male	Male	-0.028 (0.754)	-1.010 (0.526)		
Meerts 20		females	Female	-0.041 (0.435)	-0.263 (0.450)		
Meerts 20		males	Male	-0.152 (0.435)	-0.328 (0.460)		
Meerts 20	GD 17		Pup	-0.054 (0.803)	-0.371 (1.029)	0.051 (0.804)	
Meerts 20	GD 20		Pup	-0.077 (0.932)	-0.268 (0.933)	-0.013 (0.807)	
Vansell	133	male	Male	-0.048 (0.452)	-0.473 (0.323)	0.107 (0.339)	0.085 (0.484)

Table 4. Ranks of Lower 95 Percent Confidence Bounds of Slopes Across Activity Measures Within Data Segments (1=Smallest,...,5=Largest). Perchlorate.

Perchlorate								
Study	**Group**	**Gender**	**Category**	**TT3**	**TT4**	**TSH**	**TW**	**HT**
Hiasa	350g male	Male	Male	1	2	3	4	
Mahle	GD 20 dam	FEMALES	Female	1	2	3		
Mahle	GD 20 fet	NA	Pup	1	2	3		
Mahle	PND 10 da	Females	Female	1	2	3		
Mahle	PND 10 pu	Females	Pup	1	2	3		
Mahle	PND 10 pu	Male	Pup	1	2	3		
Siglin	14 days	FEMALES	Female	2	4	5	3	1
Siglin	14 days	Male	Male	1	2	4	3	5
Siglin	30 day re	FEMALES	Female	1	2	4	3	
Siglin	30 day re	Male	Male	1	4	3	2	
Siglin	90 days	FEMALES	Female	2	4	5	3	1
Siglin	90 days	Male	Male	1	4	2	3	5
York 2001	F1 adult	Females	Female	1	2	4	3	5
York 2001	F1 adult	Males	Male	1	2	4	3	5
York 2001	F1 pup	Females	Pup	1	2	3	4	5
York 2001	F1 pup	Males	Pup	1	2	3	4	5
York 2001	F2 pup	Females	Pup	1	3	2	4	5
York 2001	F2 pup	Males	Pup	1	3	2	4	5
York 2001	P adult	Females	Female	1	2	3	4	5
York 2001	P adult	Males	Male	1	2	4	3	5
York 2003	DG 21 fet	NA	Pup	3	2	5	4	1
York 2004	F1 PUPS	NA	Pup	4	3	2	1	5

Table 5. Ranks of Lower 95 Percent Confidence Bounds of Slopes Across Activity Measures Within Data Segments (1=Smallest,...,5=Largest). PTU.

PTU 1 1								
Study	**Group**	**Gender**	**Category**	**TT3**	**TT4**	**TSH**	**TW**	**HT**
Cho	28days	females	Female	2	1	3		4
Cho	28days	males	Male	3	2	4		1
HOOD	21 days	NA	Male	3	4	2	1	
Mellert		Females	Female	1	2	4	3	5
Mellert		Males	Male	2	4	5	3	1
O'Connor	Group One	NA	Male	3	5	4	2	1

Table 6. Ranks of Lower 95 Percent Confidence Bounds of Slopes Across Activity Measures Within Data Segments (1=Smallest,...,5=Largest). PCB.

PCB							
Study	**Group**	**Gender**	**Category**	**TT3**	**TT4**	**TSH**	**TW**
Bowers	dams	female	Female	1	3	2	
Bowers	offspring	NA	Pup	1	3	2	
Desaulniers		male	Male	2	3	1	
Kato		NA	Male	3	4	1	2
Kodavanti		male	Male	1	2		
Meerts 2002	GD 17		Pup	3	1	2	
Meerts 2002	GD 20		Pup	1	3	2	
Meerts 2004		females	Female	1	2		
Meerts 2004		males	Male	1	2		
Vansell	133	male	Male	2	4	3	1

Table 7. Analysis of Variance of Ranks of Lower 95 Percent Confidence Bounds of Slopes Across Chemicals, Animal Categories, Chemicals, and Activity Measures.

Covariance Parameter Estimates

Cov Parm	Estimate
group_id	0
Residual	1.1967

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Category	2	96	0.05	0.9533
Measure	4	96	6.29	0.0002
Chemical	2	96	0.33	0.7169
Category*Measure	8	96	0.94	0.4902
Chemical*Measure	7	96	3.99	0.0007

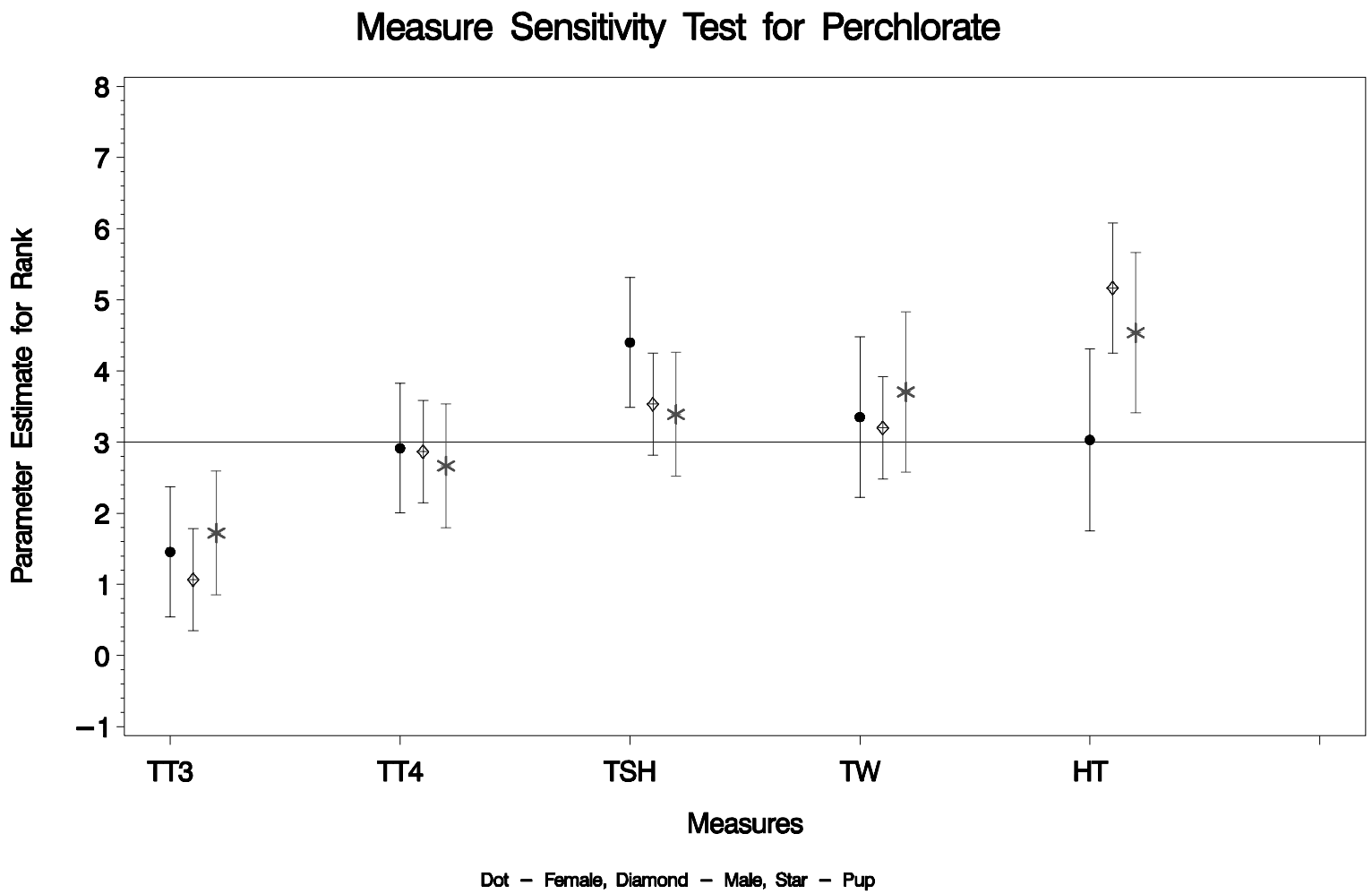


Figure 1. Least Squares Means and 95 Percent Confidence Intervals on Ranks of Lower 95 Percent Confidence Bounds of Slopes. By Animal Category and Activity Measure. Perchlorate.

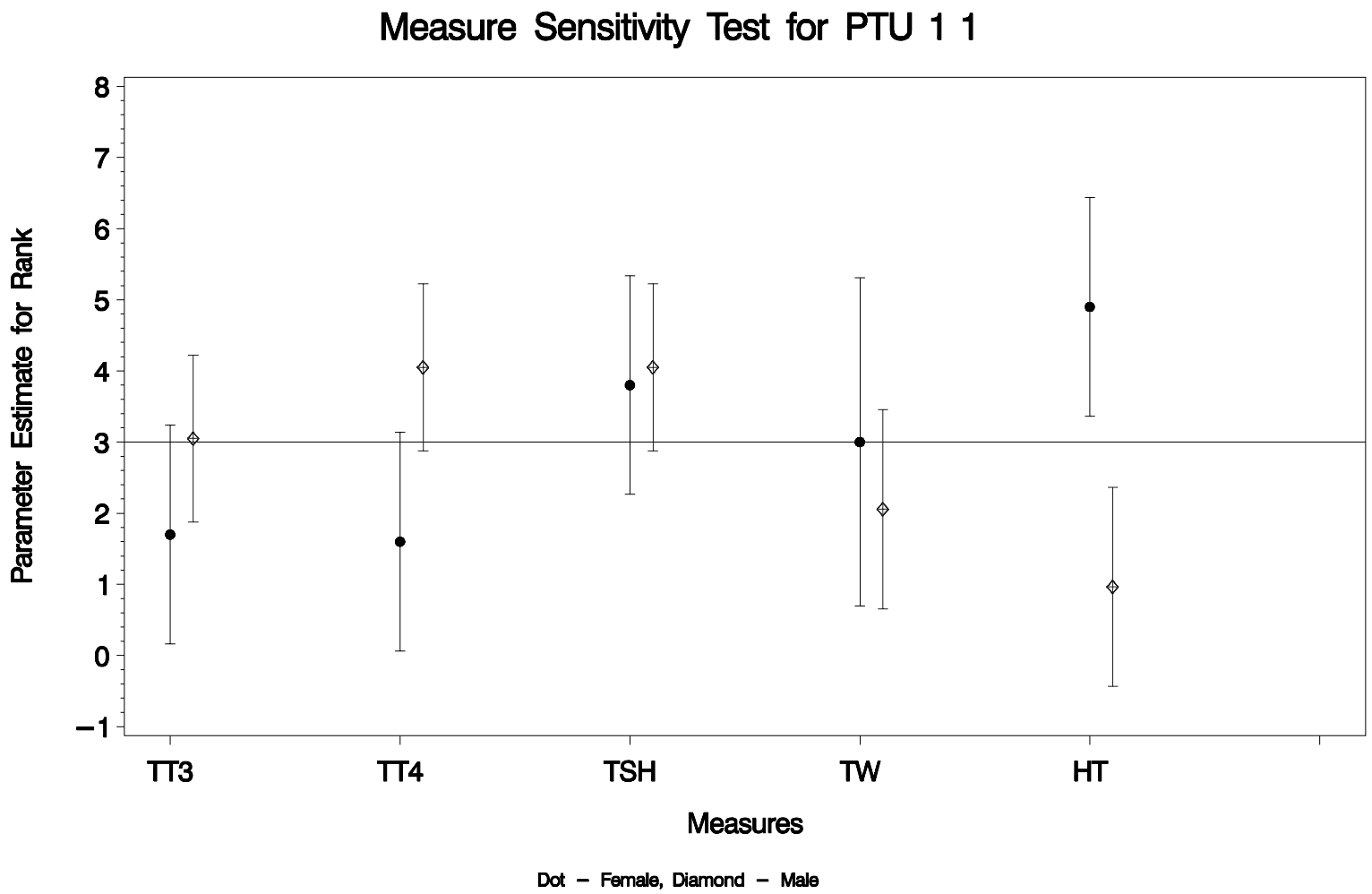


Figure 2. Least Squares Means and 95 Percent Confidence Intervals on Ranks of Lower 95 Percent Confidence Bounds of Slopes. By Animal Category and Activity Measure. PTU.

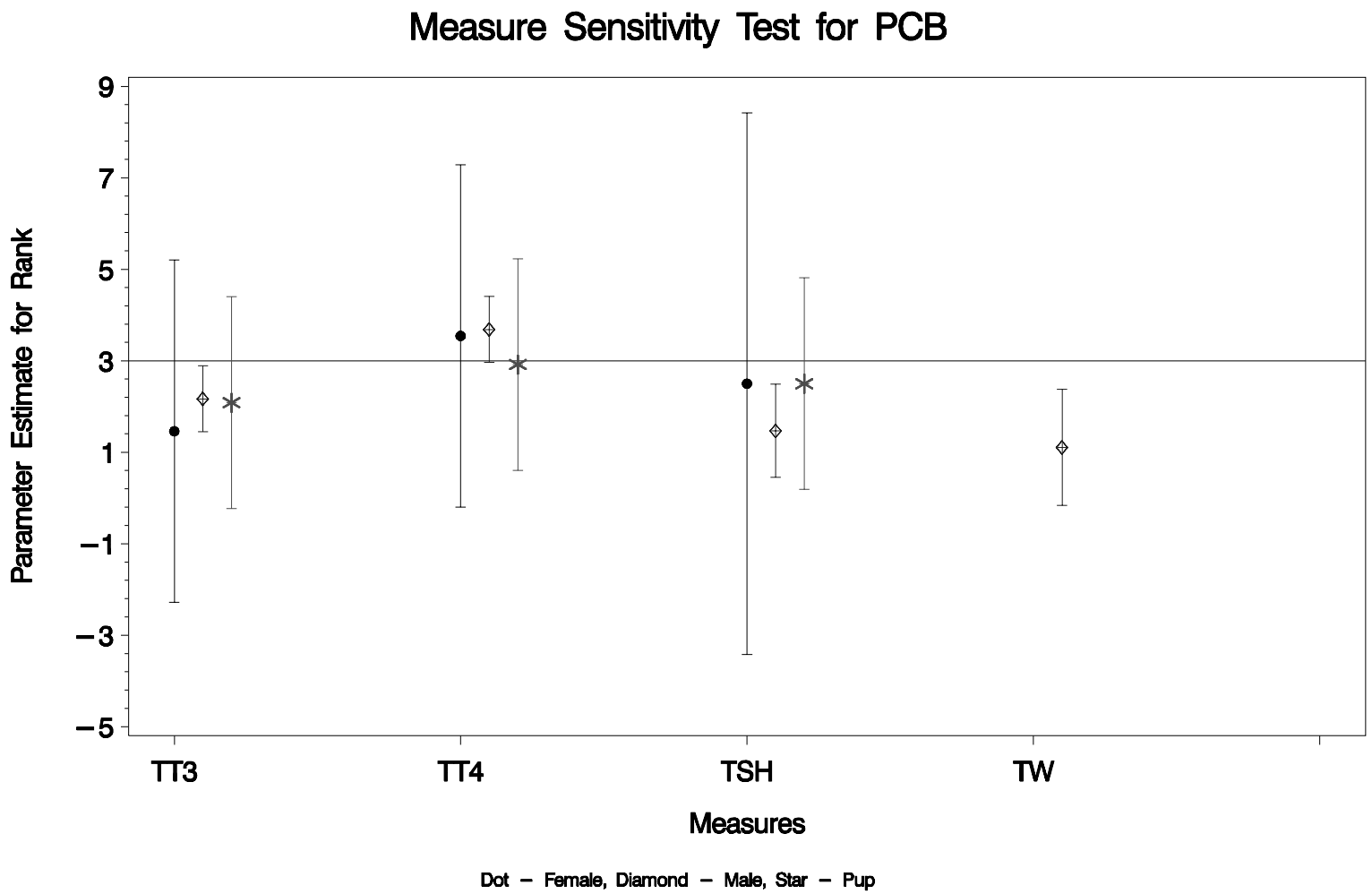


Figure 3. Least Squares Means and 95 Percent Confidence Intervals on Ranks of Lower 95 Percent Confidence Bounds of Slopes. By Animal Category and Activity Measure. PCB.

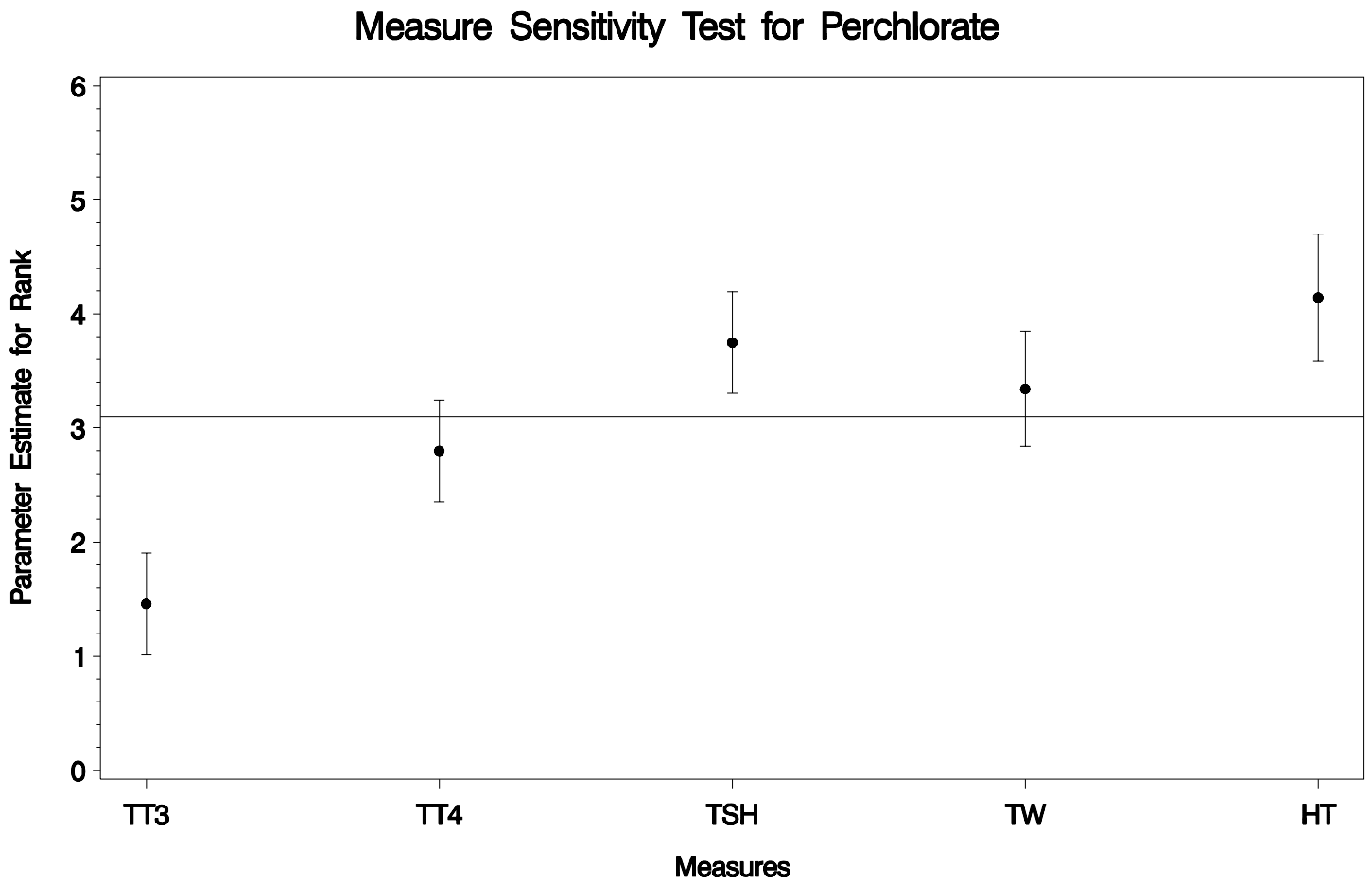


Figure 4. Least Squares Means and 95 Percent Confidence Intervals on Ranks of Lower 95 Percent Confidence Bounds of Slopes. By Activity Measure Averaged Across Animal Categories. Perchlorate.

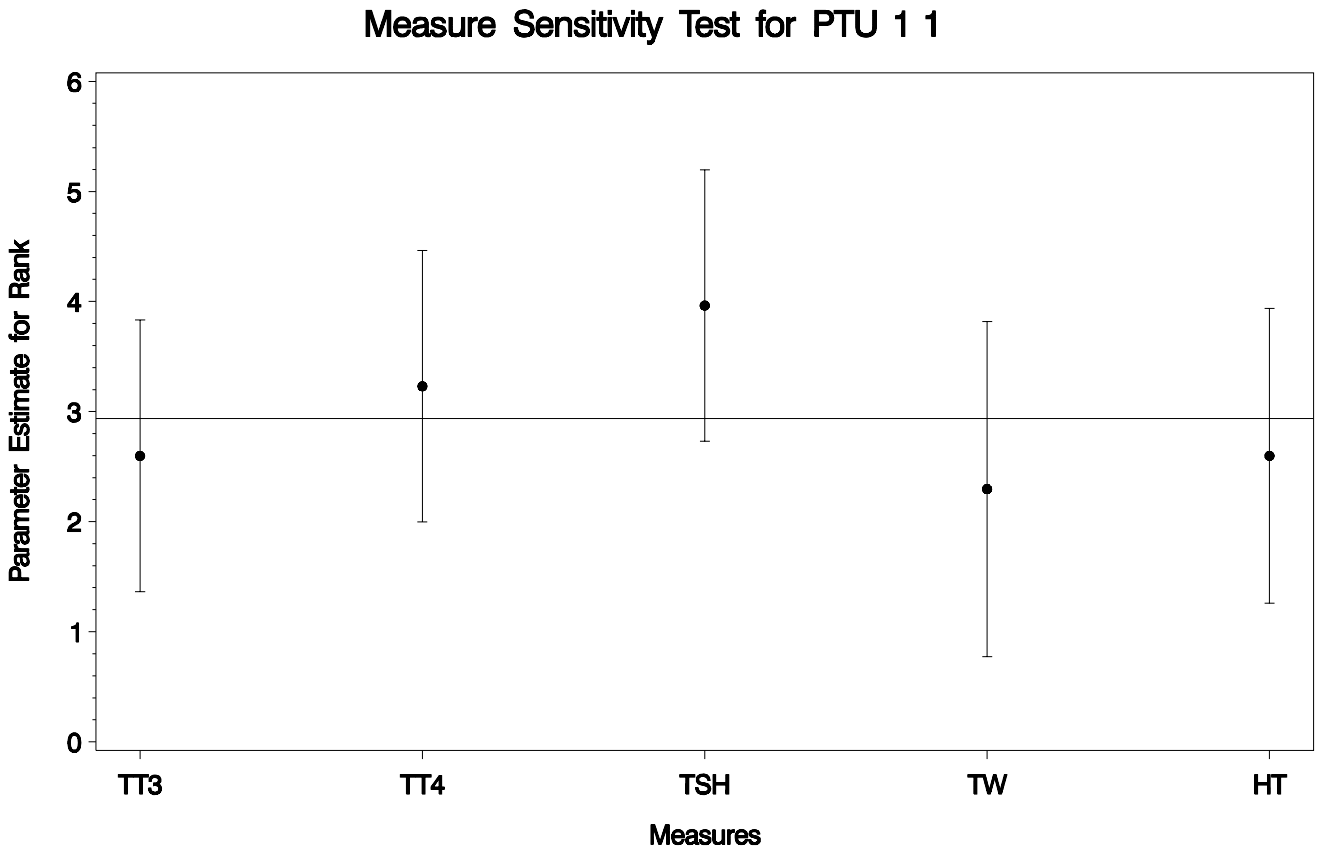


Figure 5. Least Squares Means and 95 Percent Confidence Intervals on Ranks of Lower 95 Percent Confidence Bounds of Slopes. By Activity Measure Averaged Across Animal Categories. PTU.

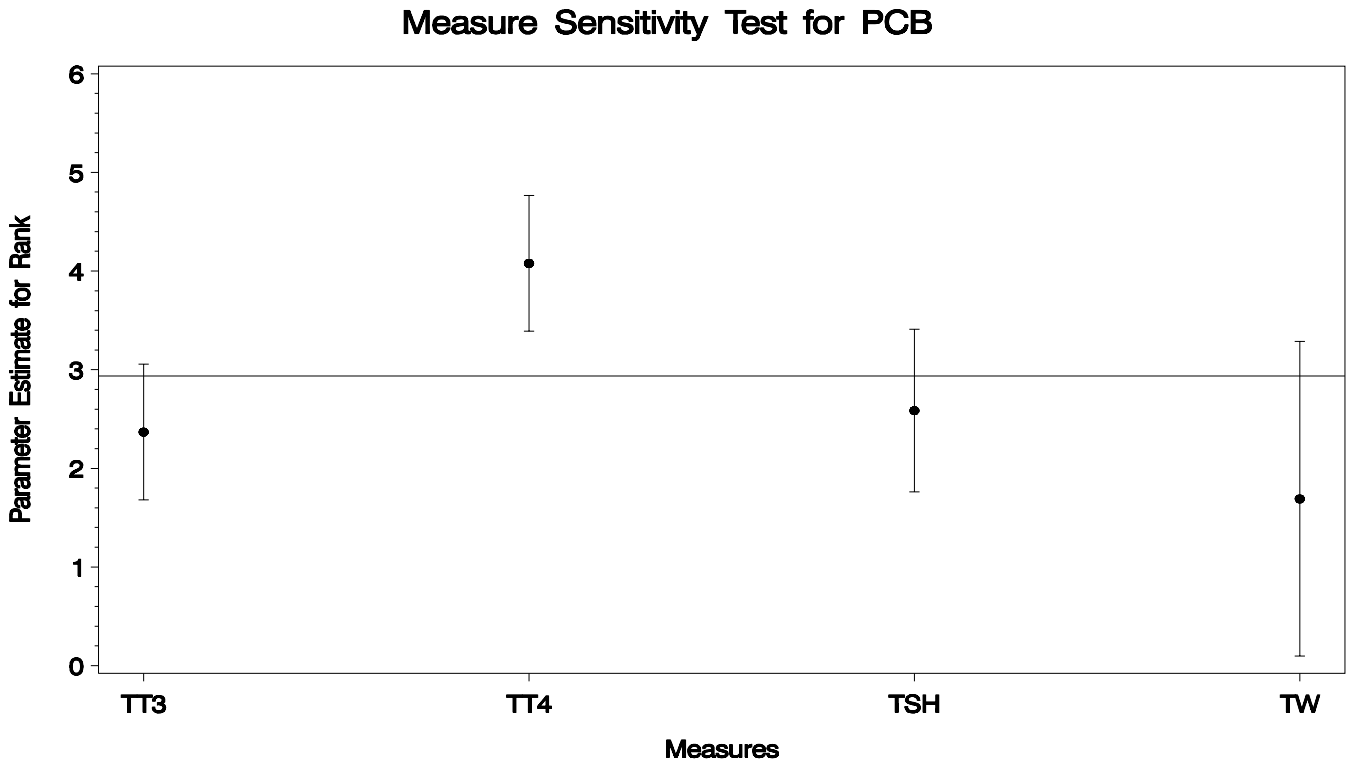


Figure 6. Least Squares Means and 95 Percent Confidence Intervals on Ranks of Lower 95 Percent Confidence Bounds of Slopes. By Activity Measure Averaged Across Animal Categories. PCB.

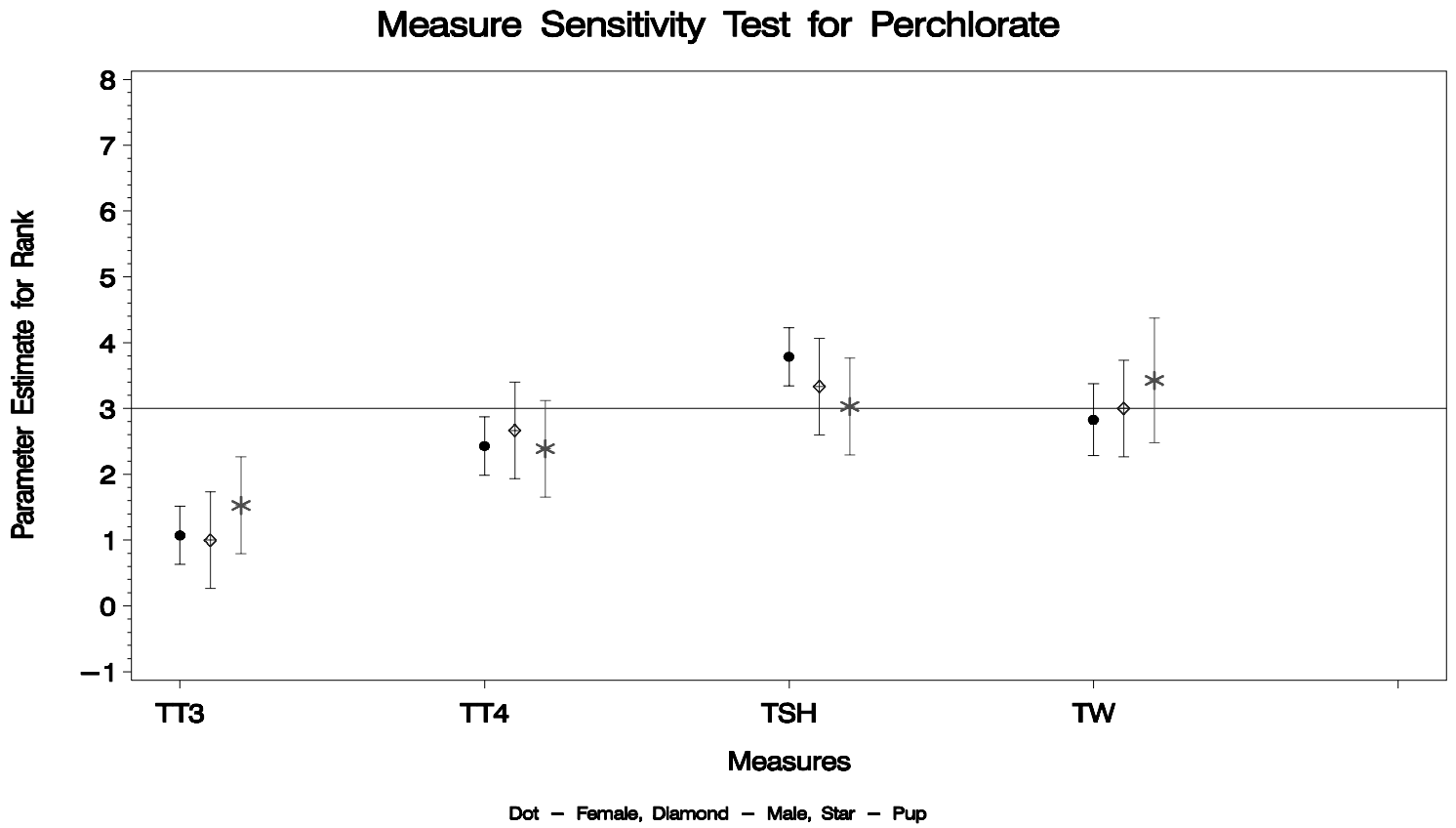


Figure 7. Least Squares Means and 95 Percent Confidence Intervals on Ranks of Lower 95 Percent Confidence Bounds of Slopes Omitting Hypertrophy. By Animal Category and Activity Measure. Perchlorate.

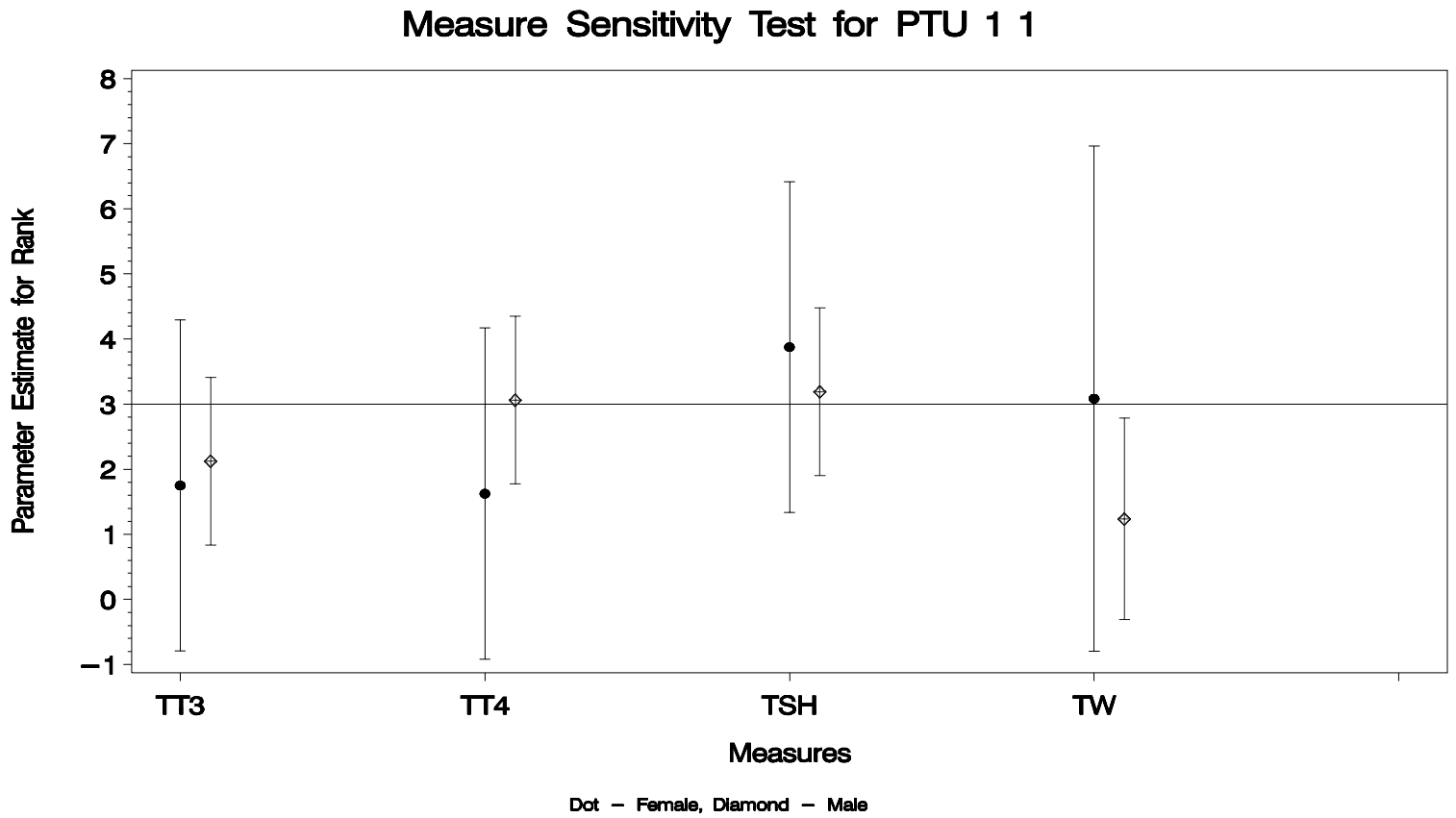


Figure 8. Least Squares Means and 95 Percent Confidence Intervals on Ranks of Lower 95 Percent Confidence Bounds of Slopes Omitting Hypertrophy. By Animal Category and Activity Measure. PTU.

Table 8. Ranks of Lower 95 Percent Confidence Bounds of Slopes Across Activity Measures Within Data Segments (1=Smallest,...,5=Largest). Sensitivity Analysis Based on Normalized Untransformed Data. Perchlorate.

Perchlorate								
Study	**Group**	**Gender**	**Category**	**TT3**	**TT4**	**TSH**	**TW**	**HT**
Hiasa	350g male	Male	Male	4	2	1	3	
Mahle	GD 20 dam	FEMALES	Female	3	1	2		
Mahle	GD 20 fet	NA	Pup	3	1	2		
Mahle	PND 10 da	Females	Female	2	1	3		
Mahle	PND 10 pu	Females	Pup	2	1	3		
Mahle	PND 10 pu	Male	Pup	3	2	1		
Siglin	14 days	FEMALES	Female	4	3	5	2	1
Siglin	14 days	Male	Male	1	4	3	2	5
Siglin	30 day re	FEMALES	Female	2	1	4	3	
Siglin	30 day re	Male	Male	3	1	4	2	
Siglin	90 days	FEMALES	Female	5	3	2	4	1
Siglin	90 days	Male	Male	1	3	4	2	5
York 2001	F1 adult	Females	Female	3	1	4	2	5
York 2001	F1 adult	Males	Male	2	4	1	3	5
York 2001	F1 pup	Females	Pup	1	2	4	3	5
York 2001	F1 pup	Males	Pup	4	2	1	3	5
York 2001	F2 pup	Females	Pup	4	1	2	3	5
York 2001	F2 pup	Males	Pup	3	1	2	4	5
York 2001	P adult	Females	Female	1	4	2	3	5
York 2001	P adult	Males	Male	1	4	2	3	5
York 2003	DG 21 fet	NA	Pup	5	4	3	2	1
York 2004	F1 PUPS	NA	Pup	5	3	2	4	1

Table 9. Ranks of Lower 95 Percent Confidence Bounds of Slopes Across Activity Measures Within Data Segments (1=Smallest,...,5=Largest). Sensitivity Analysis Based on Normalized Untransformed Data. PTU.

PTU 1 1								
Study	**Group**	**Gender**	**Category**	**TT3**	**TT4**	**TSH**	**TW**	**HT**
Cho	28days	females	Female	3	2	1		4
Cho	28days	males	Male	4	3	2		1
HOOD	21 days	NA	Male	3	1	4	2	
Mellert		Females	Female	2	4	1	3	5
Mellert		Males	Male	4	2	5	3	1
O'Connor	Group One	NA	Male	5	4	3	2	1

Table 10. Ranks of Lower 95 Percent Confidence Bounds of Slopes Across Activity Measures Within Data Segments (1=Smallest,...,5=Largest). Sensitivity Analysis Based on Normalized Untransformed Data. PCB.

PCB							
Study	**Group**	**Gender**	**Category**	**TT3**	**TT4**	**TSH**	**TW**
Bowers	dams	female	Female	1	3	2	
Bowers	offspring	NA	Pup	3	2	1	
Desaulniers		male	Male	3	1	2	
Kato		NA	Male	2	3	1	4
Kodavanti		male	Male	2	1		
Meerts 2002	GD 17		Pup	1	2	3	
Meerts 2002	GD 20		Pup	1	3	2	
Meerts 2004		females	Female	2	1		
Meerts 2004		males	Male	2	1		
Vansell	133	male	Male	1	3	2	4

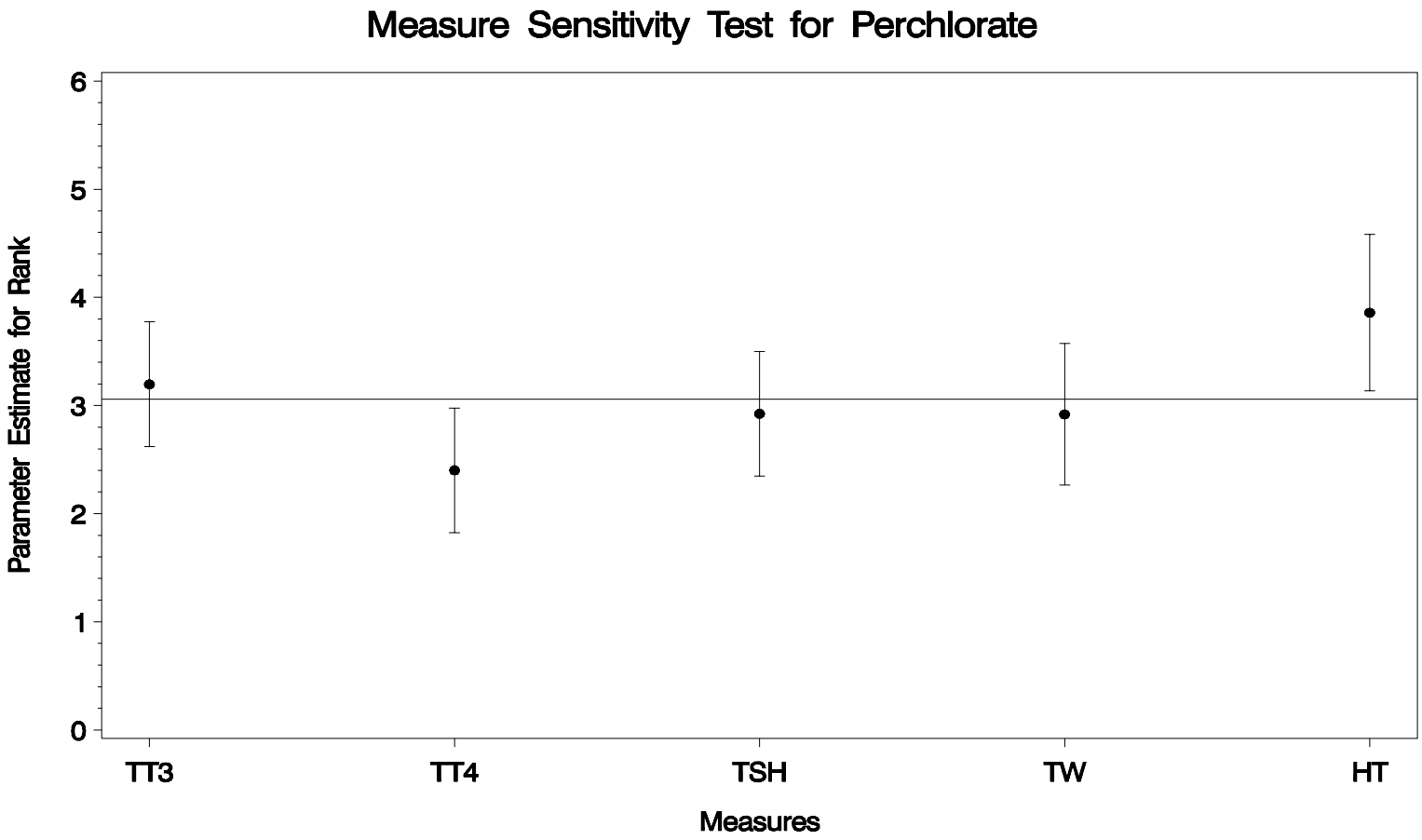


Figure 9. Least Squares Means and 95 Percent Confidence Intervals on Ranks of Lower 95 Percent Confidence Bounds of Slopes. By Activity Measure Pooled Across Animal Characteristics. Sensitivity Analysis Based on Normalized Untransformed Data. Perchlorate.

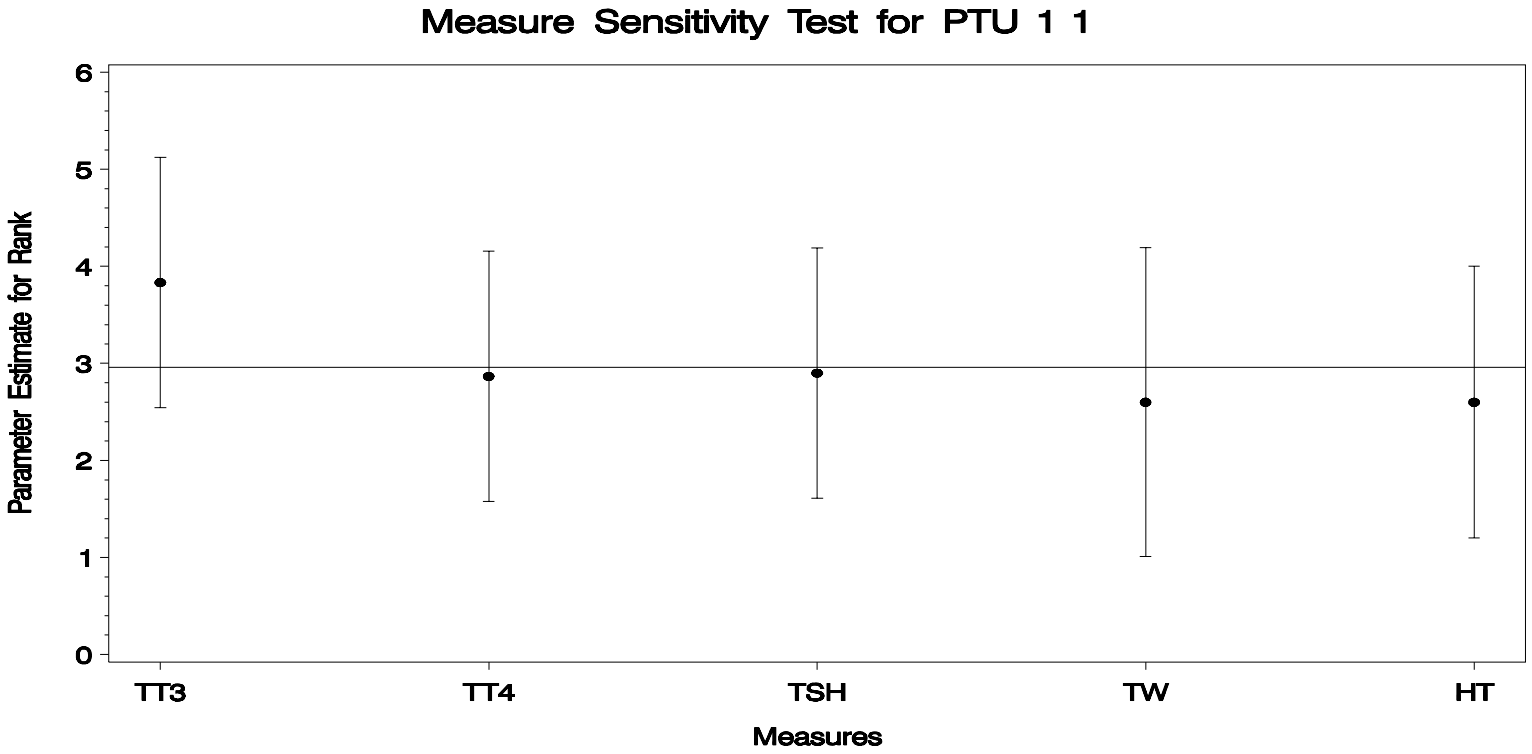


Figure 10. Least Squares Means and 95 Percent Confidence Intervals on Ranks of Lower 95 Percent Confidence Bounds of Slopes. By Activity Measure Pooled Across Animal Characteristics. Sensitivity Analysis Based on Normalized Untransformed Data. PTU.

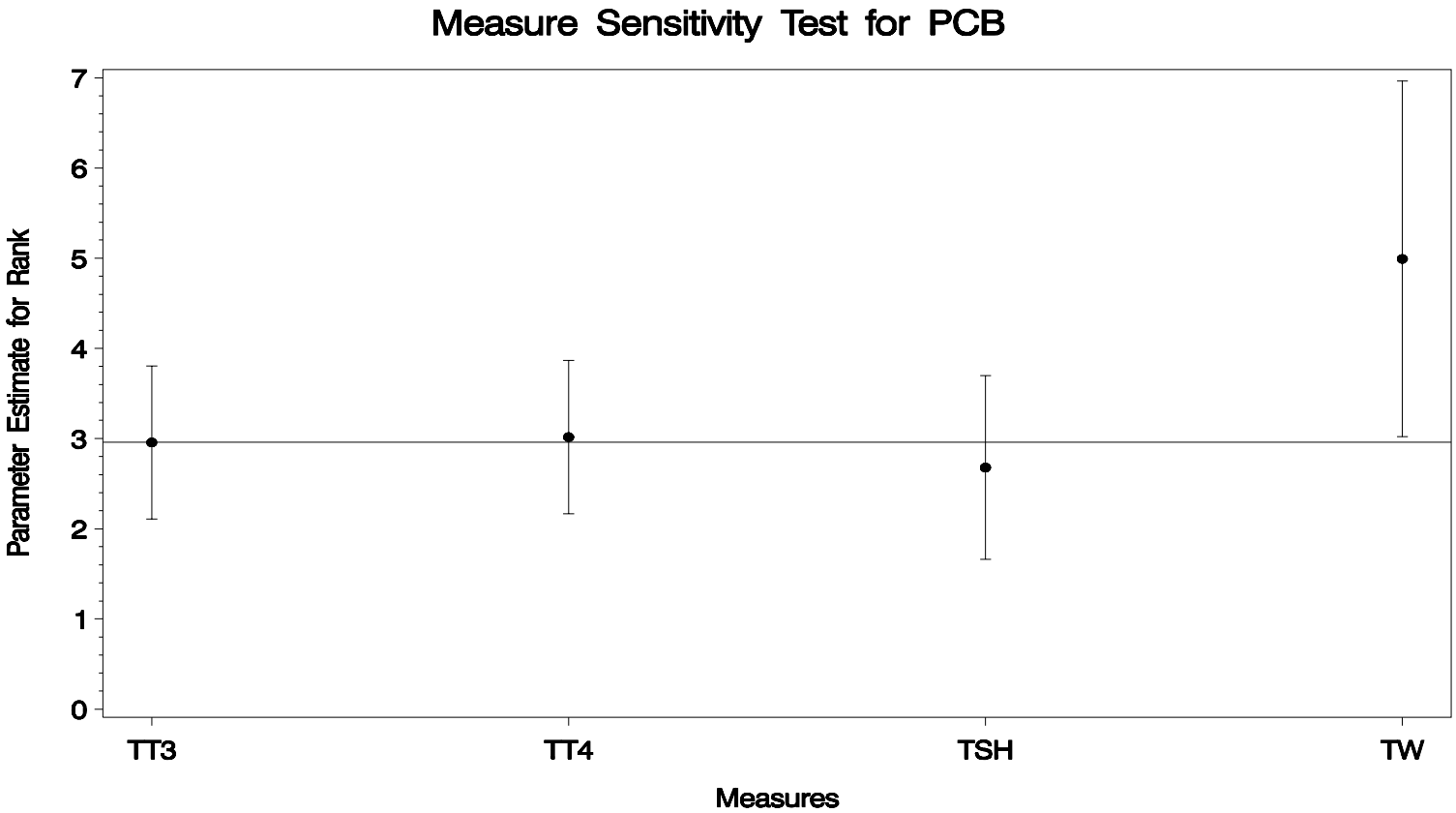
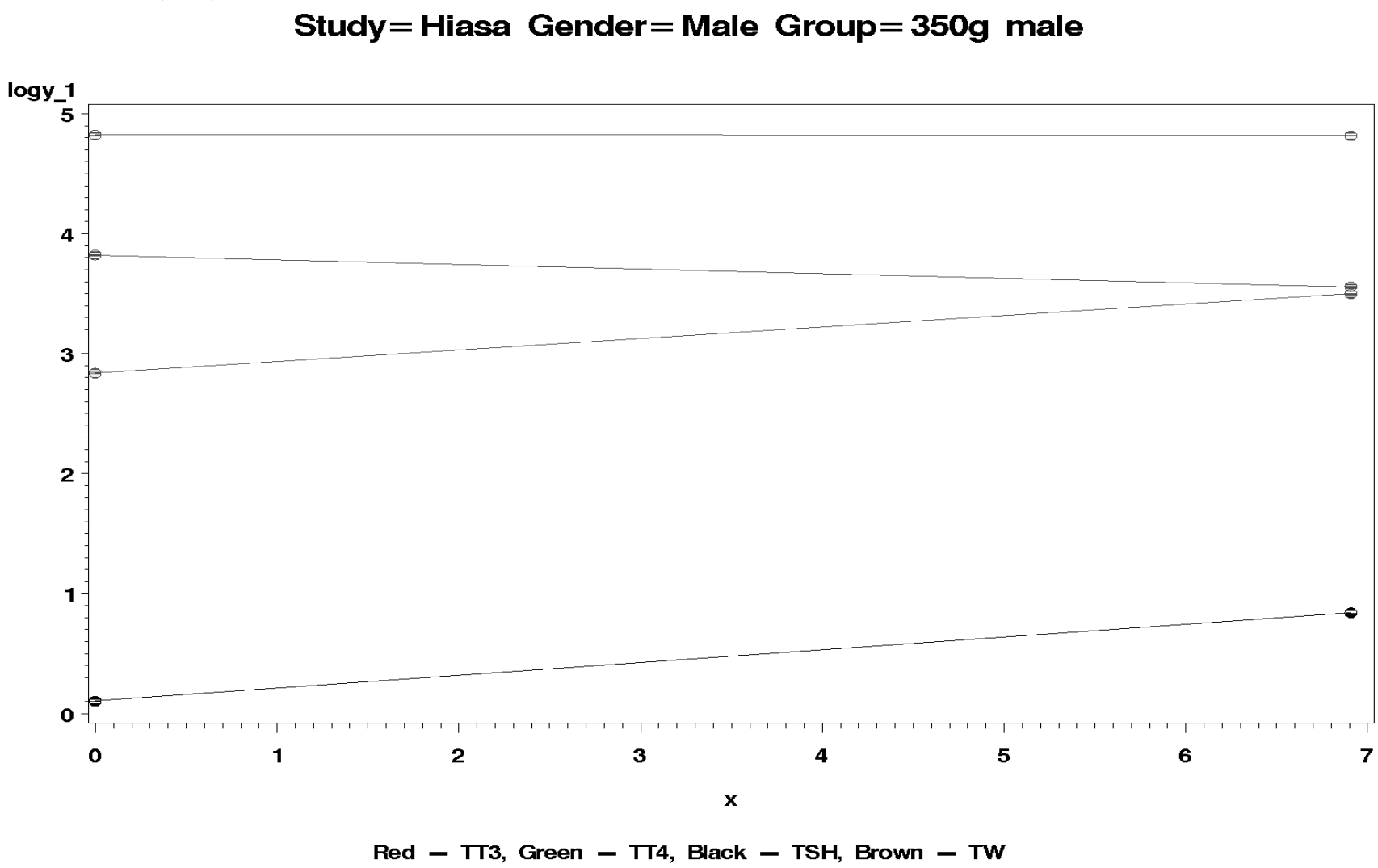


Figure 11. Least Squares Means and 95 Percent Confidence Intervals on Ranks of Lower 95 Percent Confidence Bounds of Slopes. By Activity Measure Pooled Across Animal Characteristics. Sensitivity Analysis Based on Normalized Untransformed Data. PCB.

APPENDIX I

PERCHLORATE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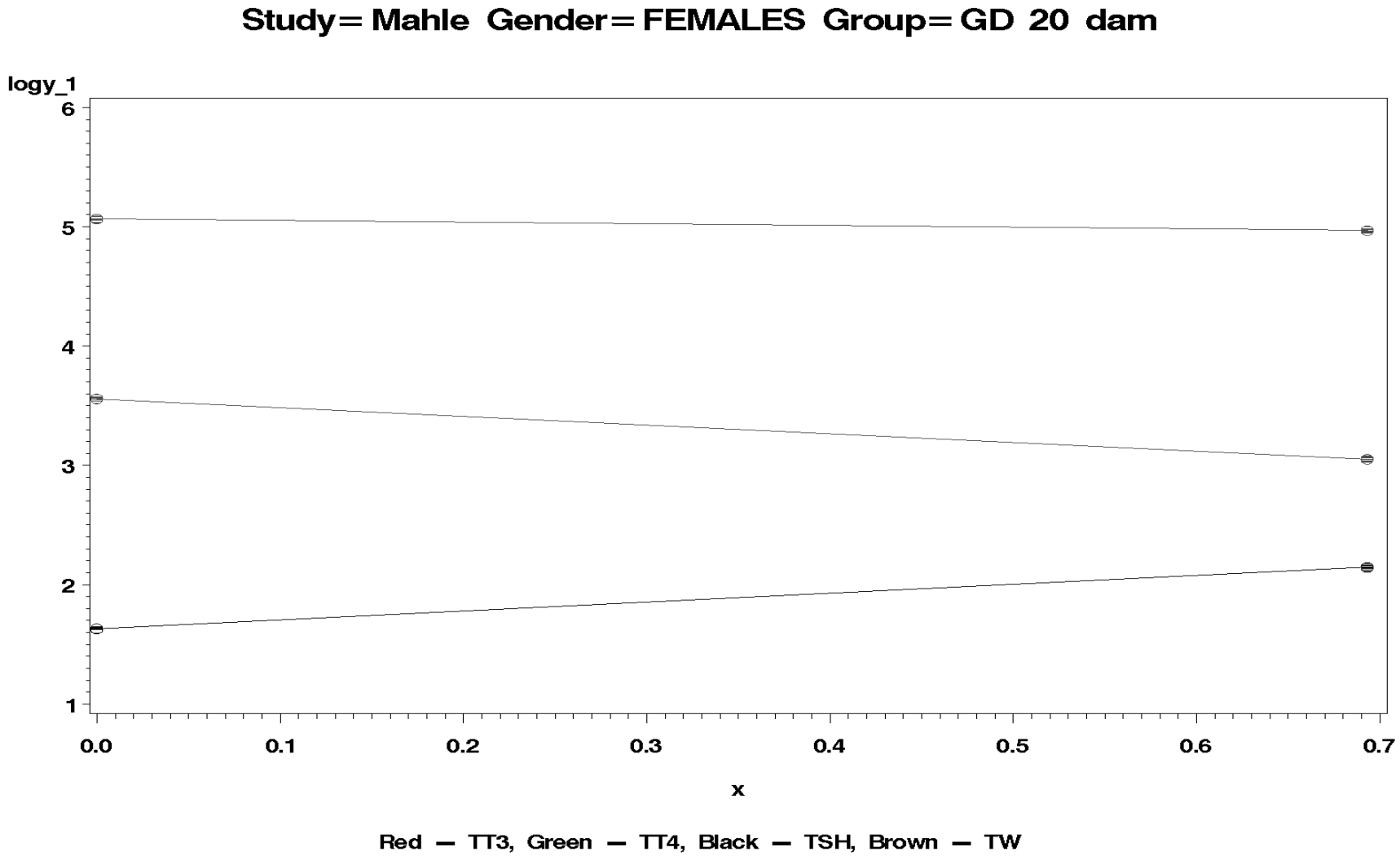
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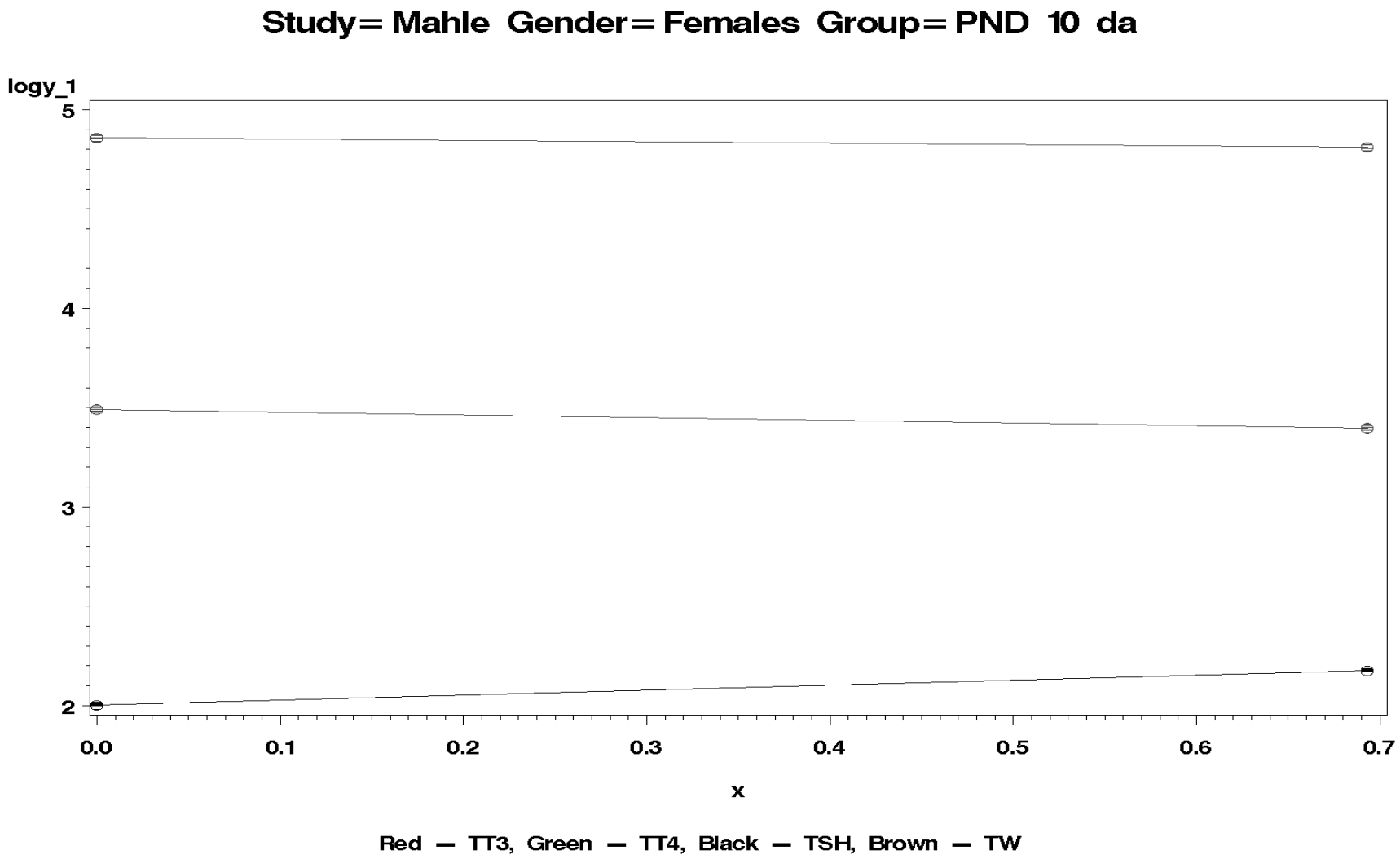
0.7
x
Red — TT3, Green — TT4, Black — TSH, Brown — TW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0.6
0.7
x
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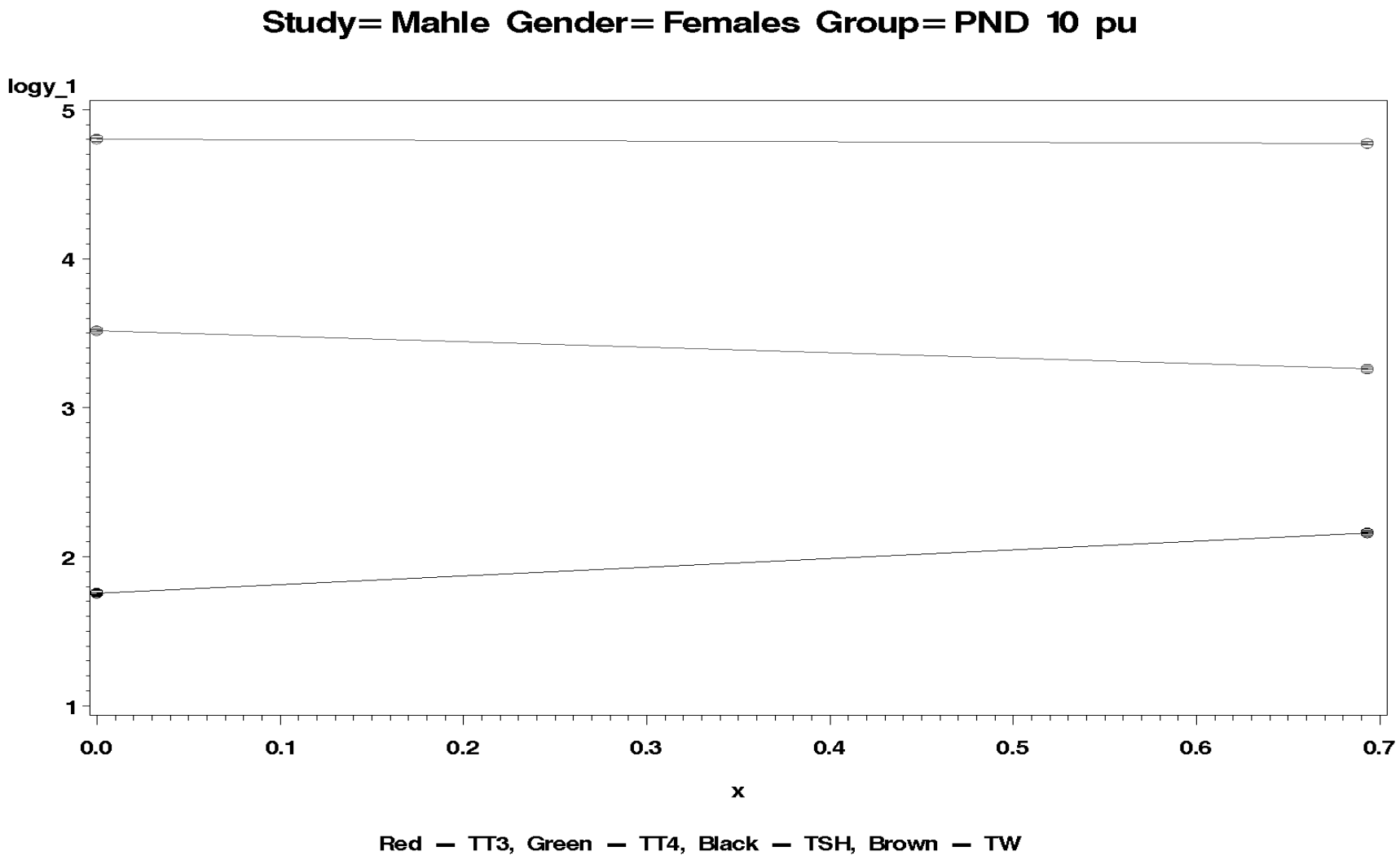
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0.5
0.6
0.7
x
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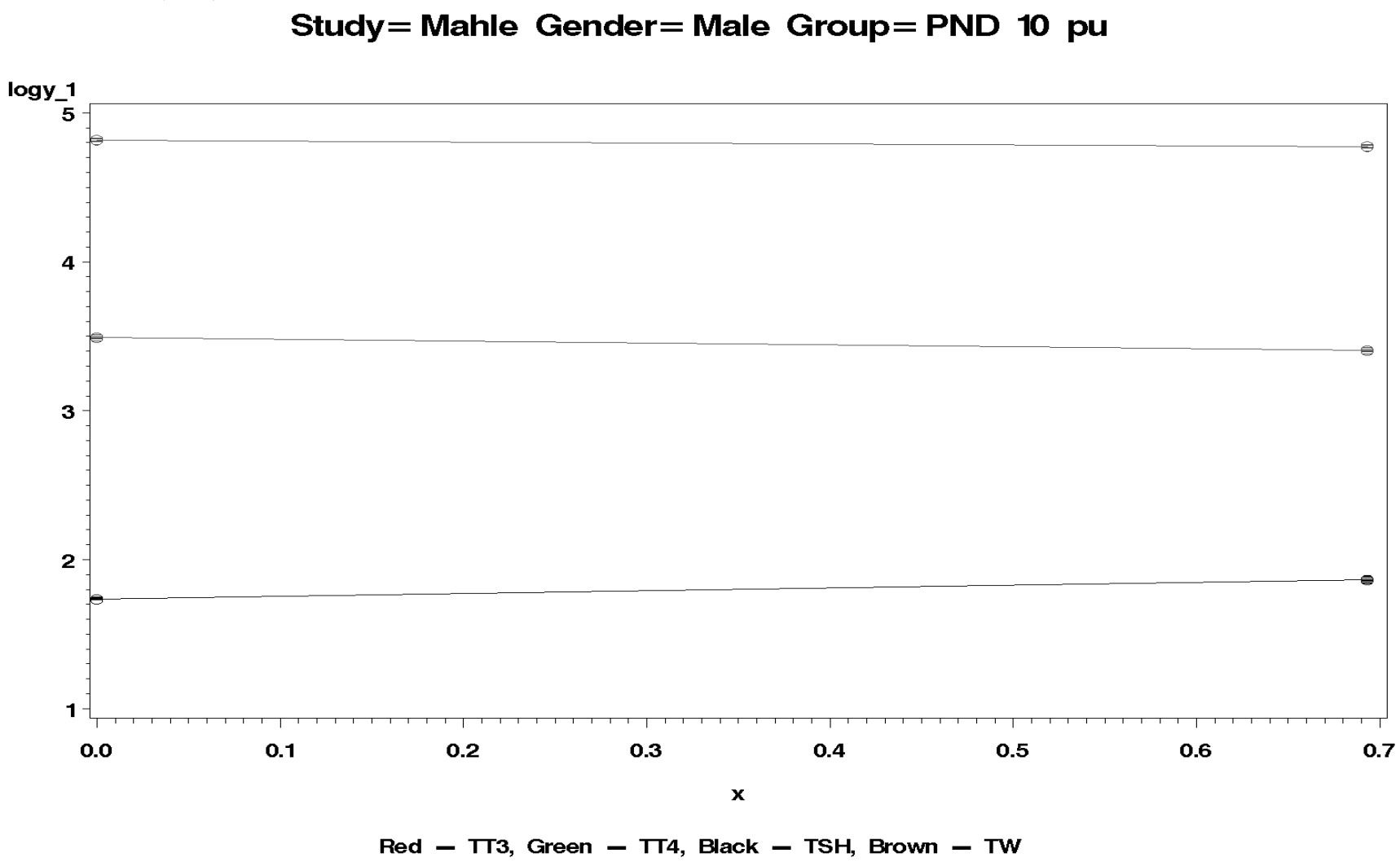
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0.5
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0.7
x
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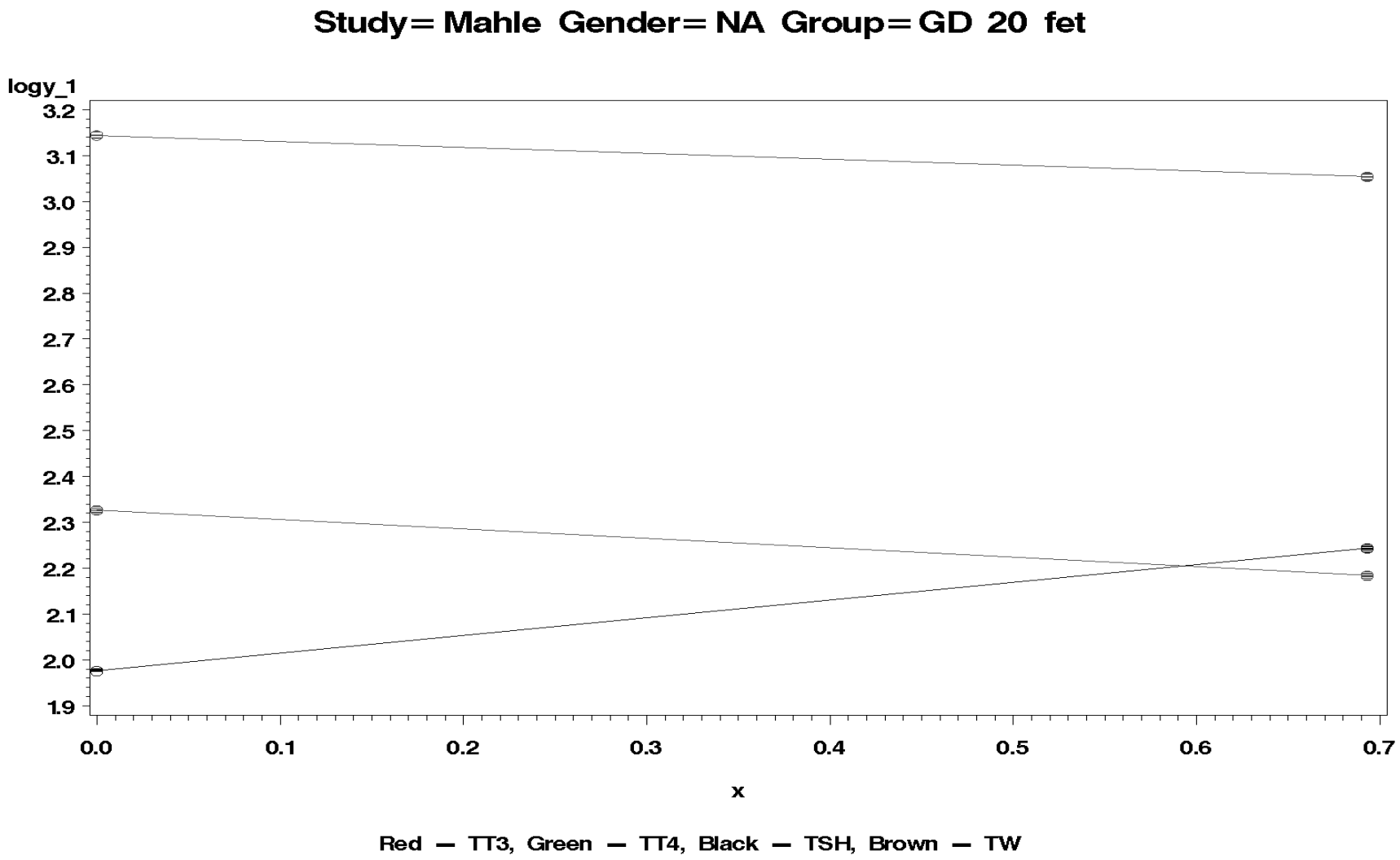
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2.8
2.7
2.6
2.5
2.4
2.3
2.2
2.1
2.0
1.9
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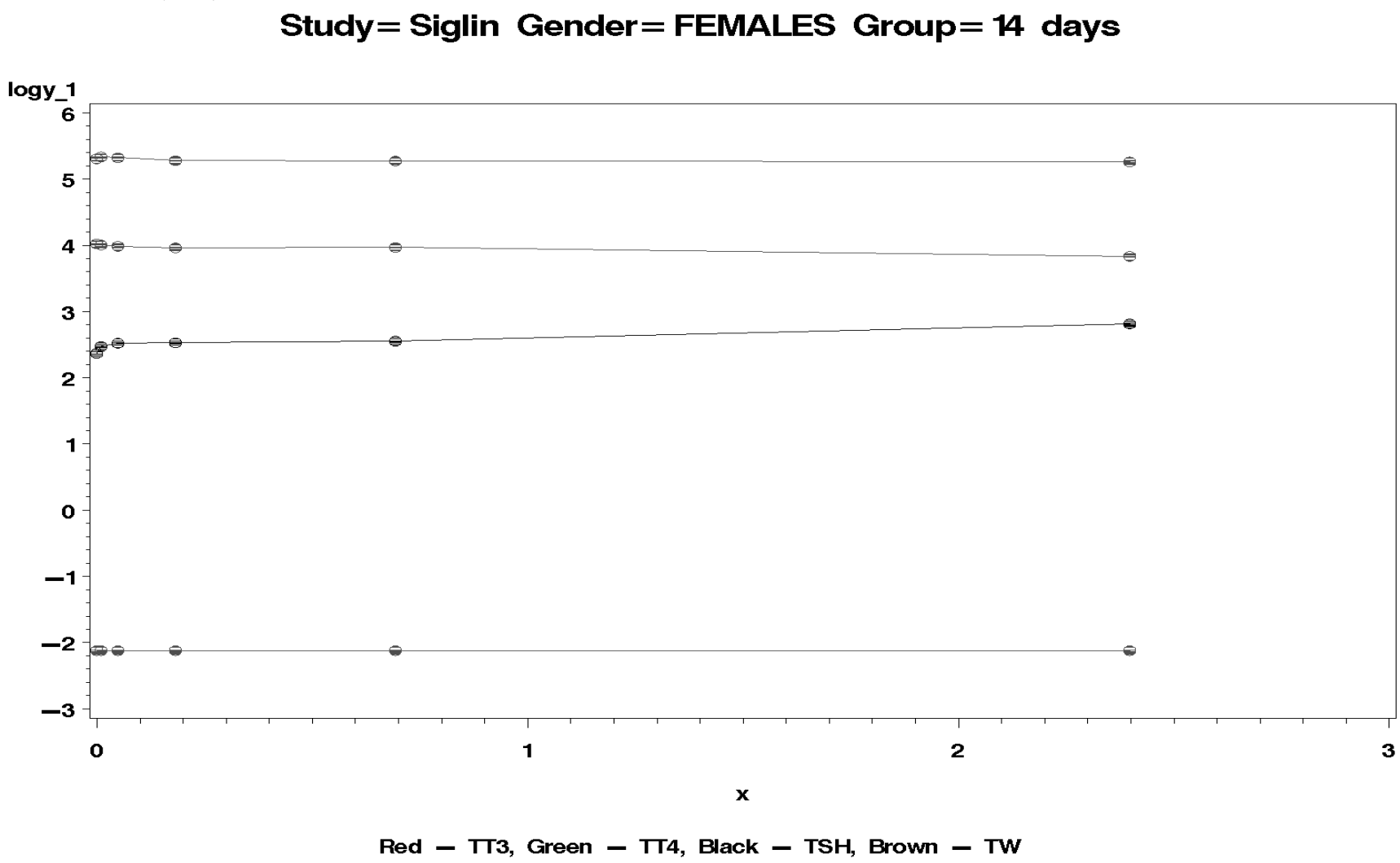
0.5
0.6
0.7
x
Red — TT3, Green — TT4, Black — TSH, Brown — TW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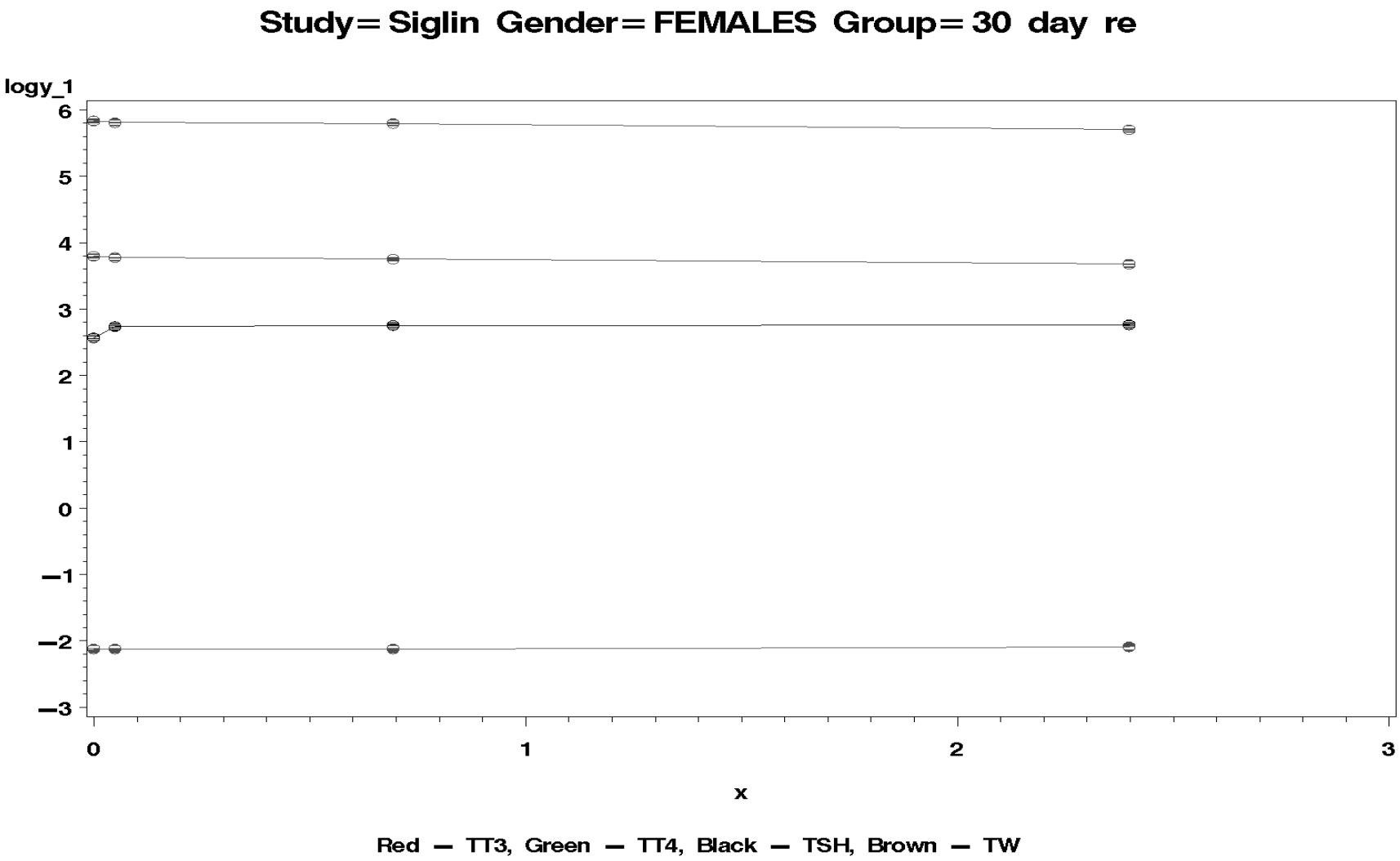
0
1
2
3
x
Red — TT3, Green — TT4, Black — TSH, Brown — TW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3
x
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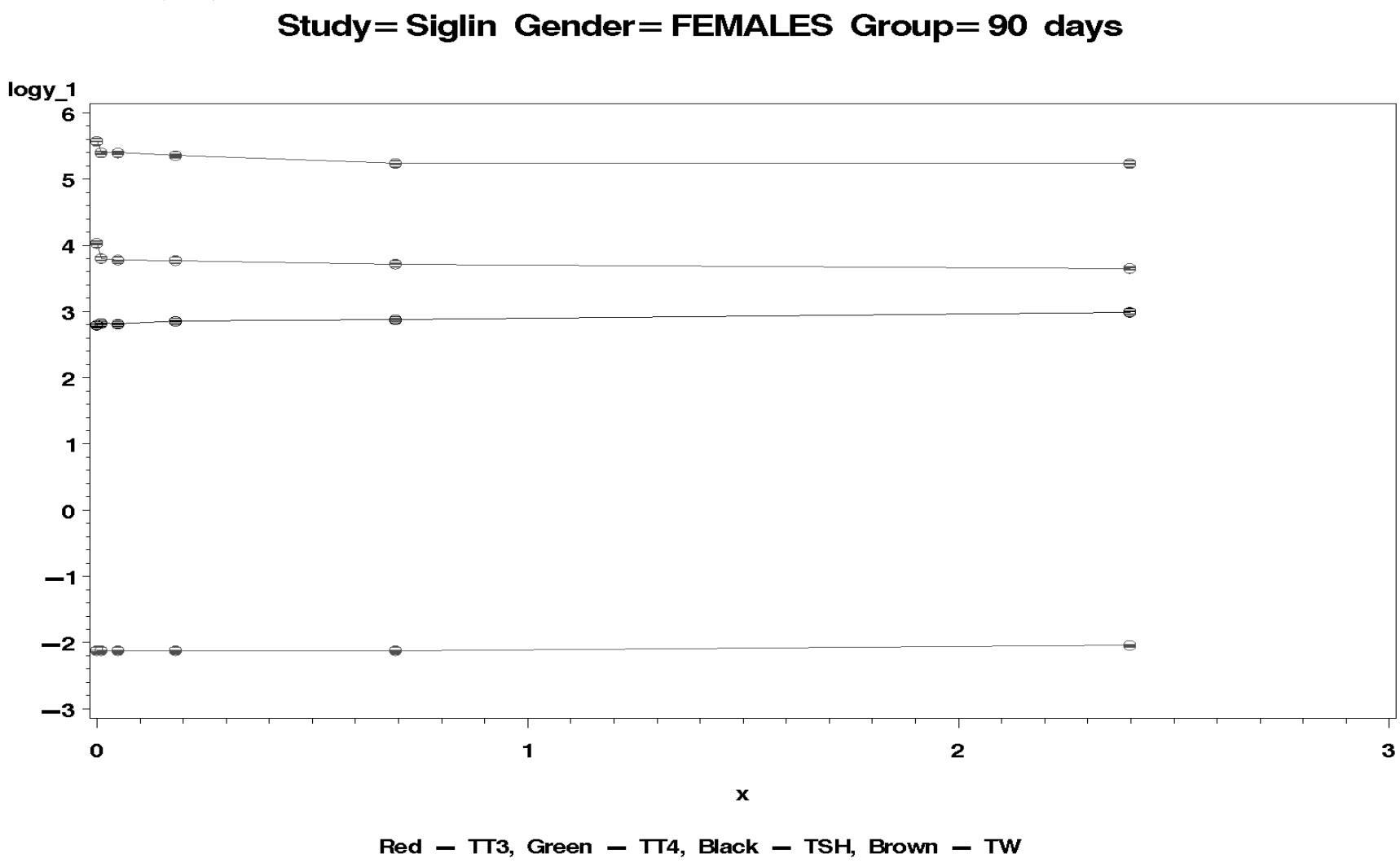
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3
x
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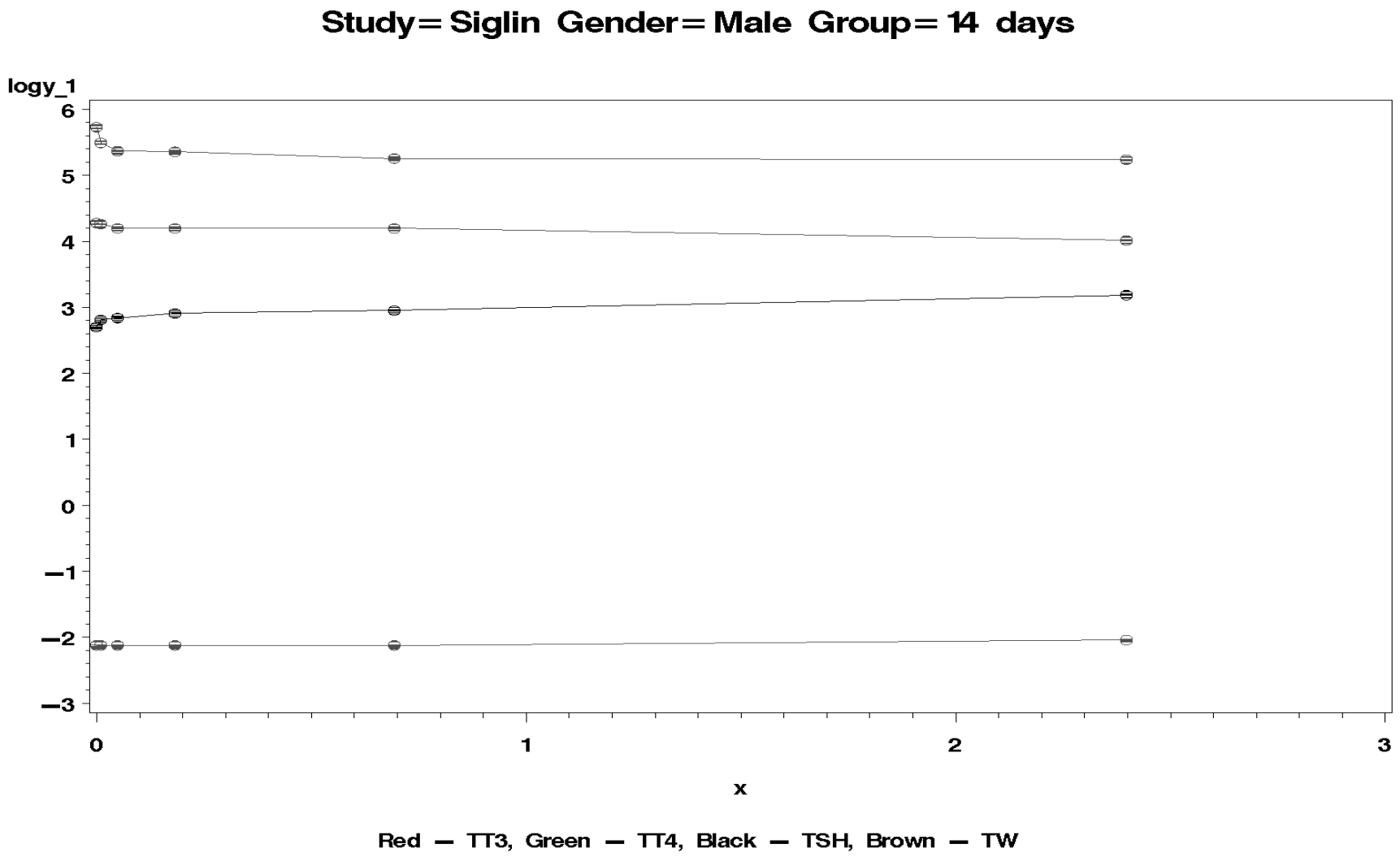
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x
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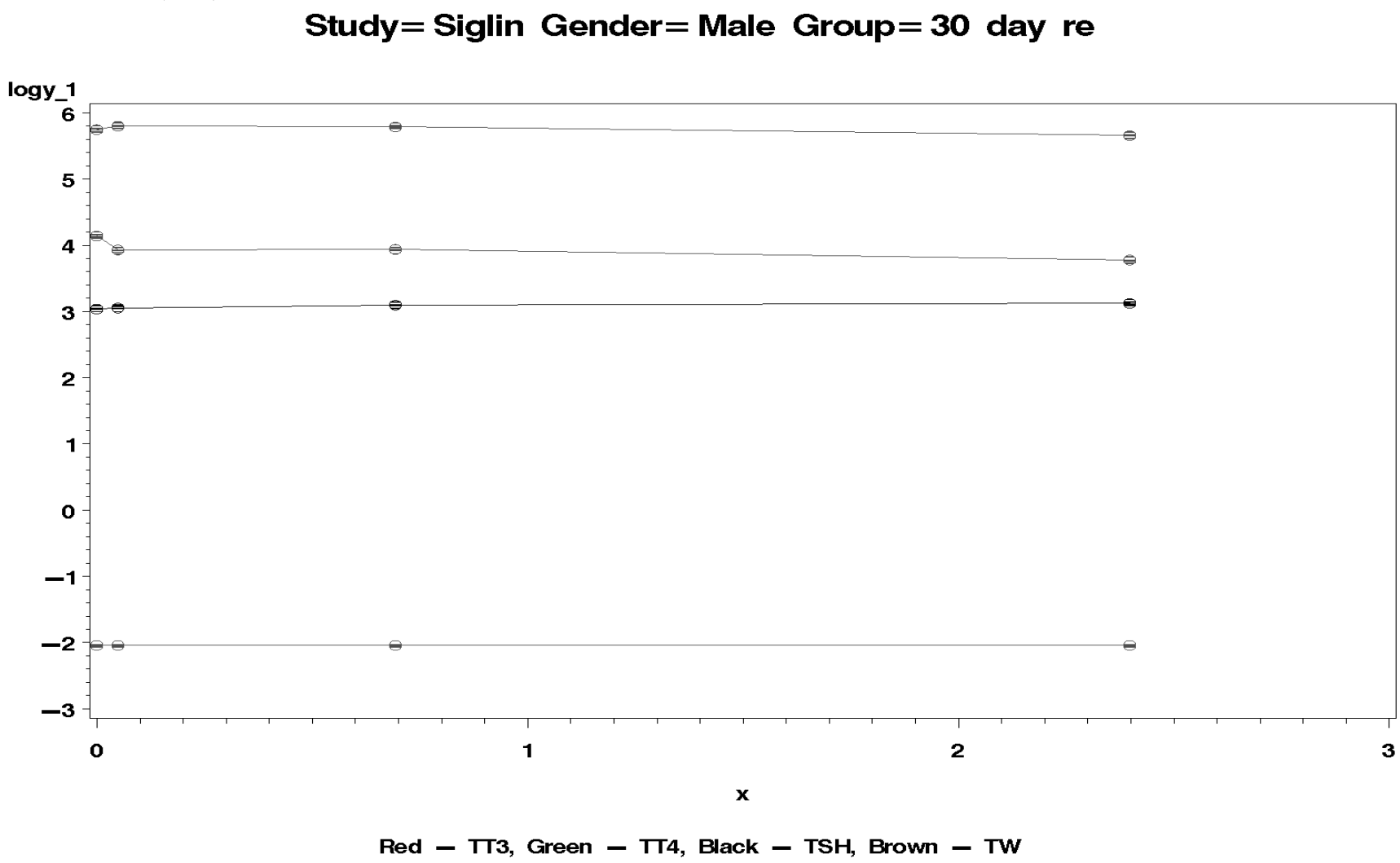
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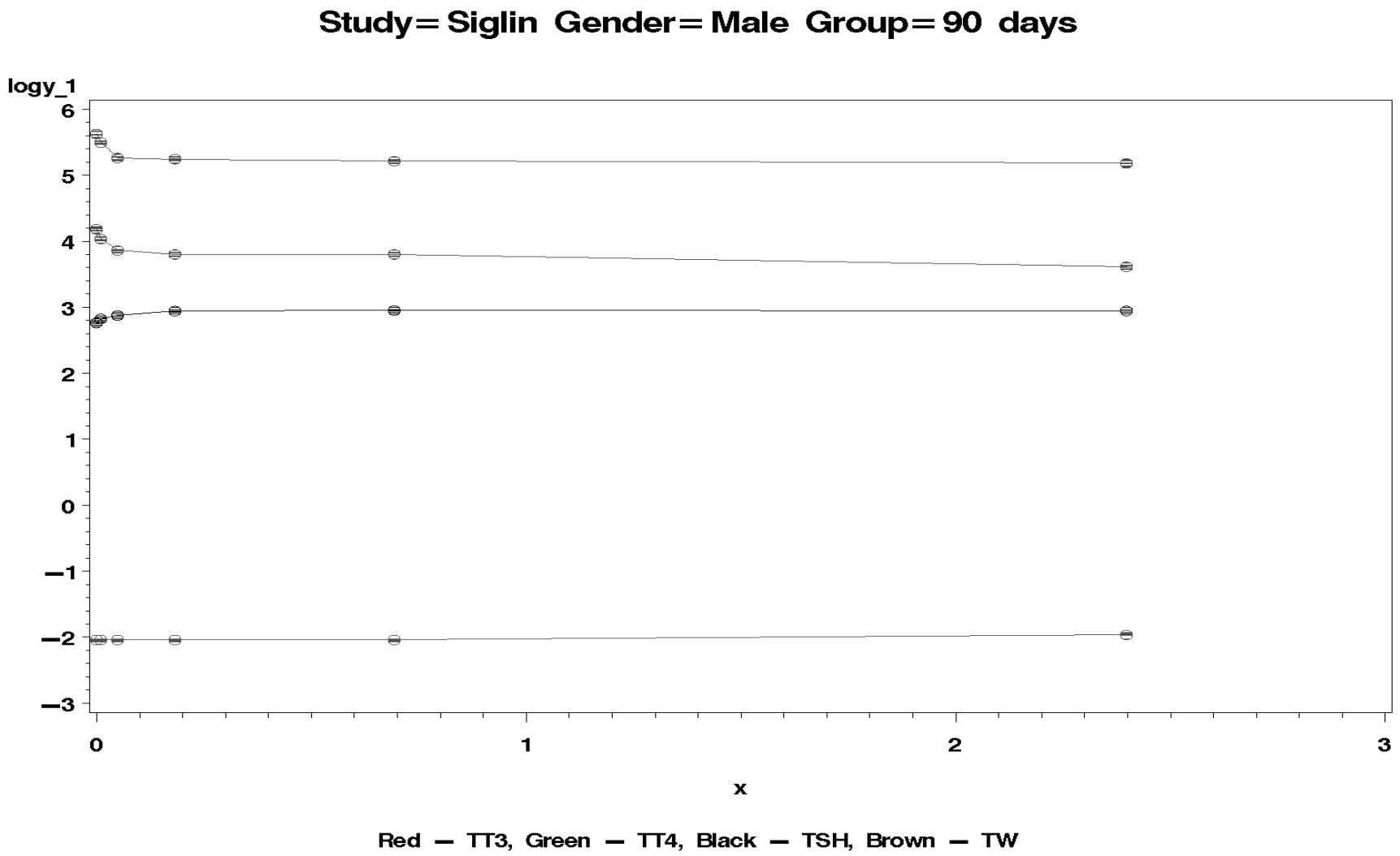
x
Red — TT3, Green — TT4, Black — TSH, Brown — TW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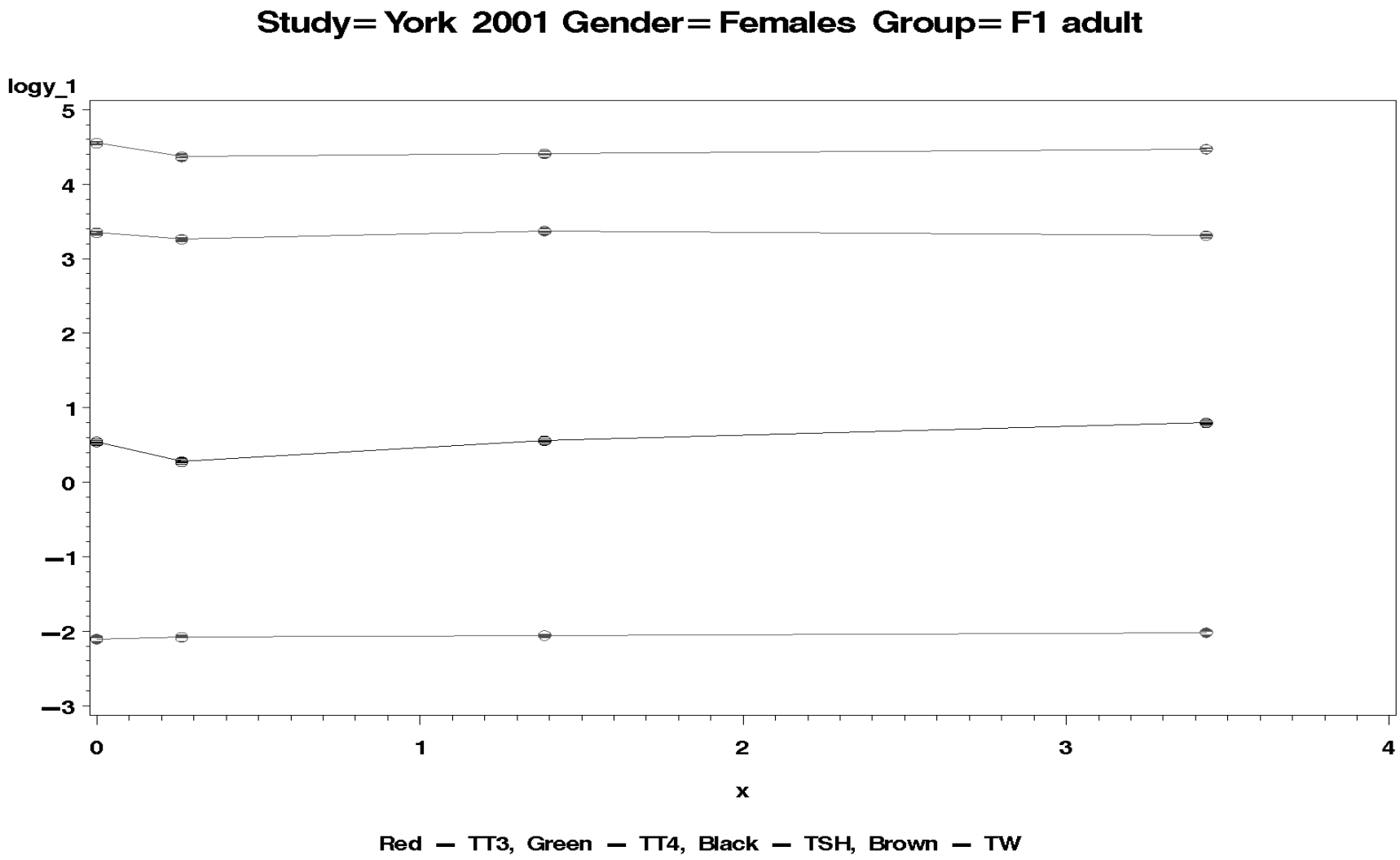
3
x
Red — TT3, Green — TT4, Black — TSH, Brown — TW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4
x
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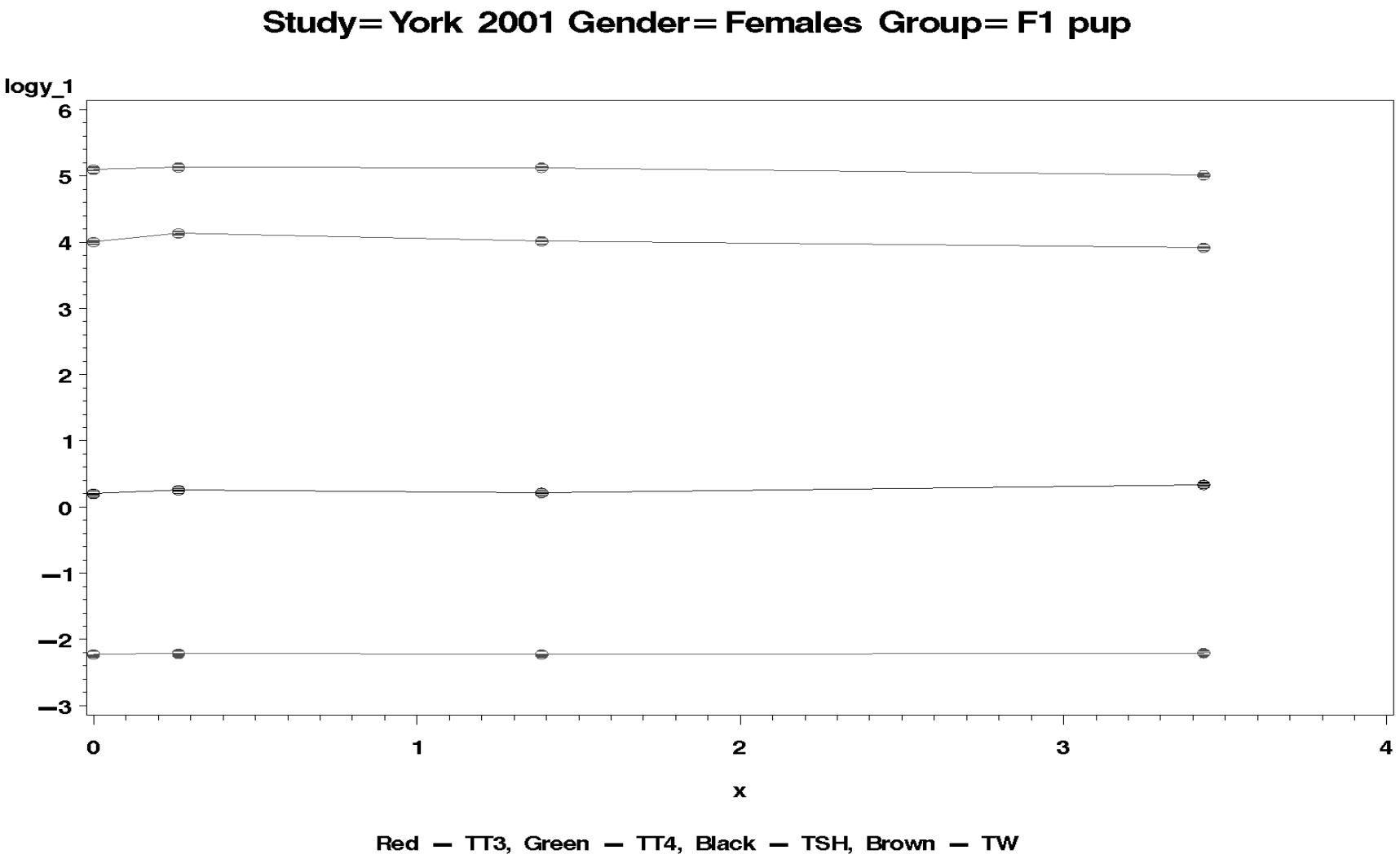
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4
x
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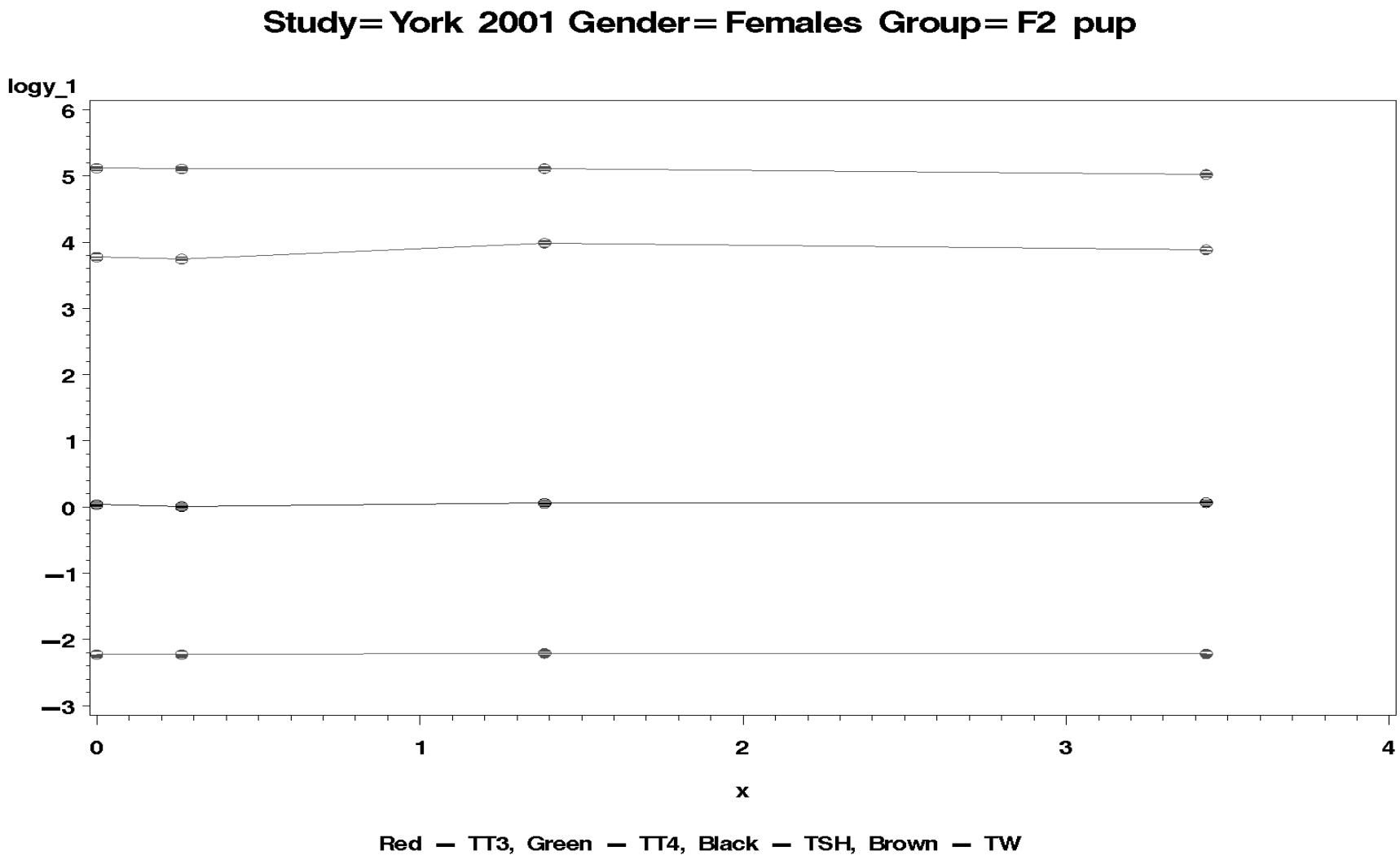
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4
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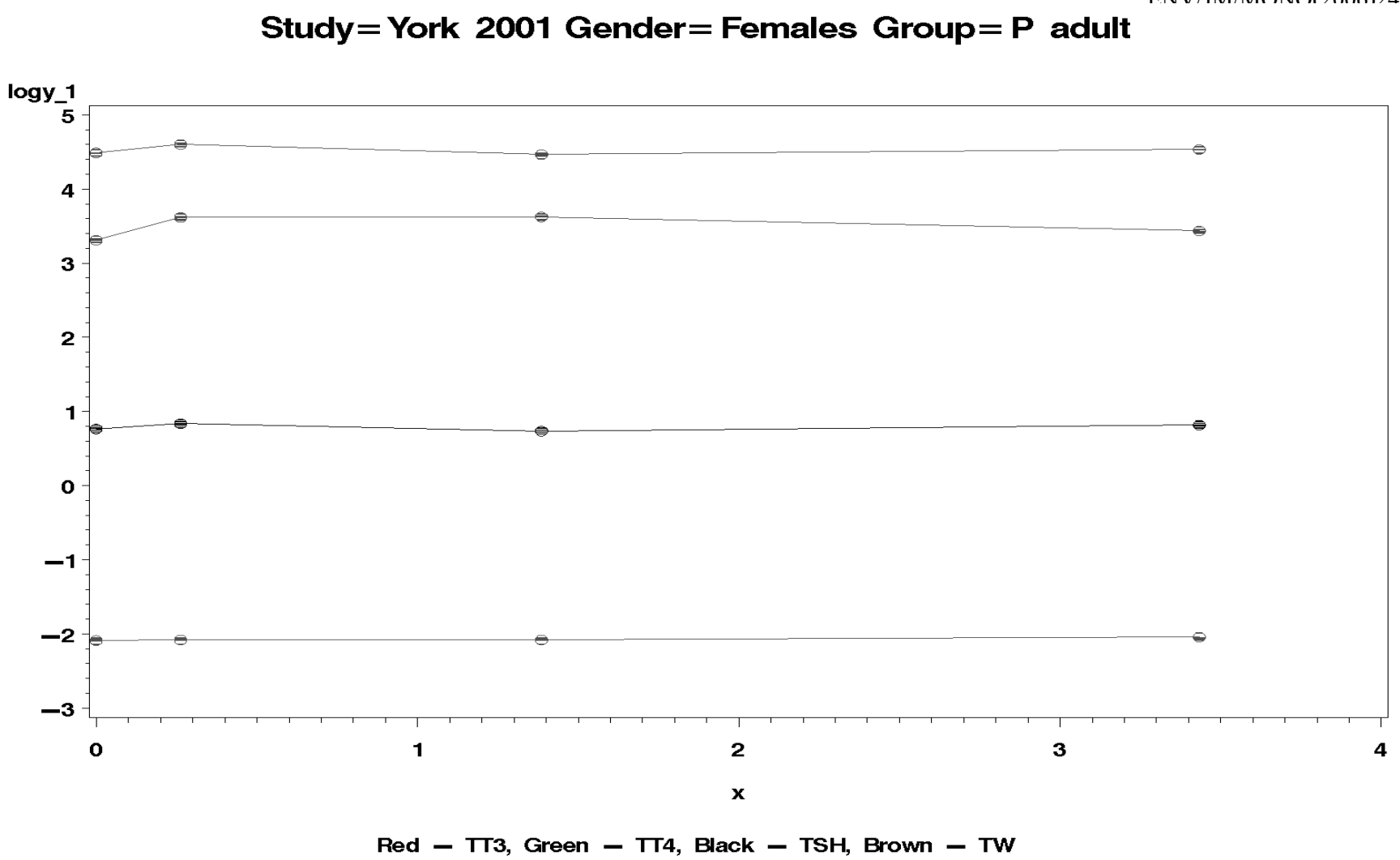
4
x
Red — TT3, Green — TT4, Black — TSH, Brown — TW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x
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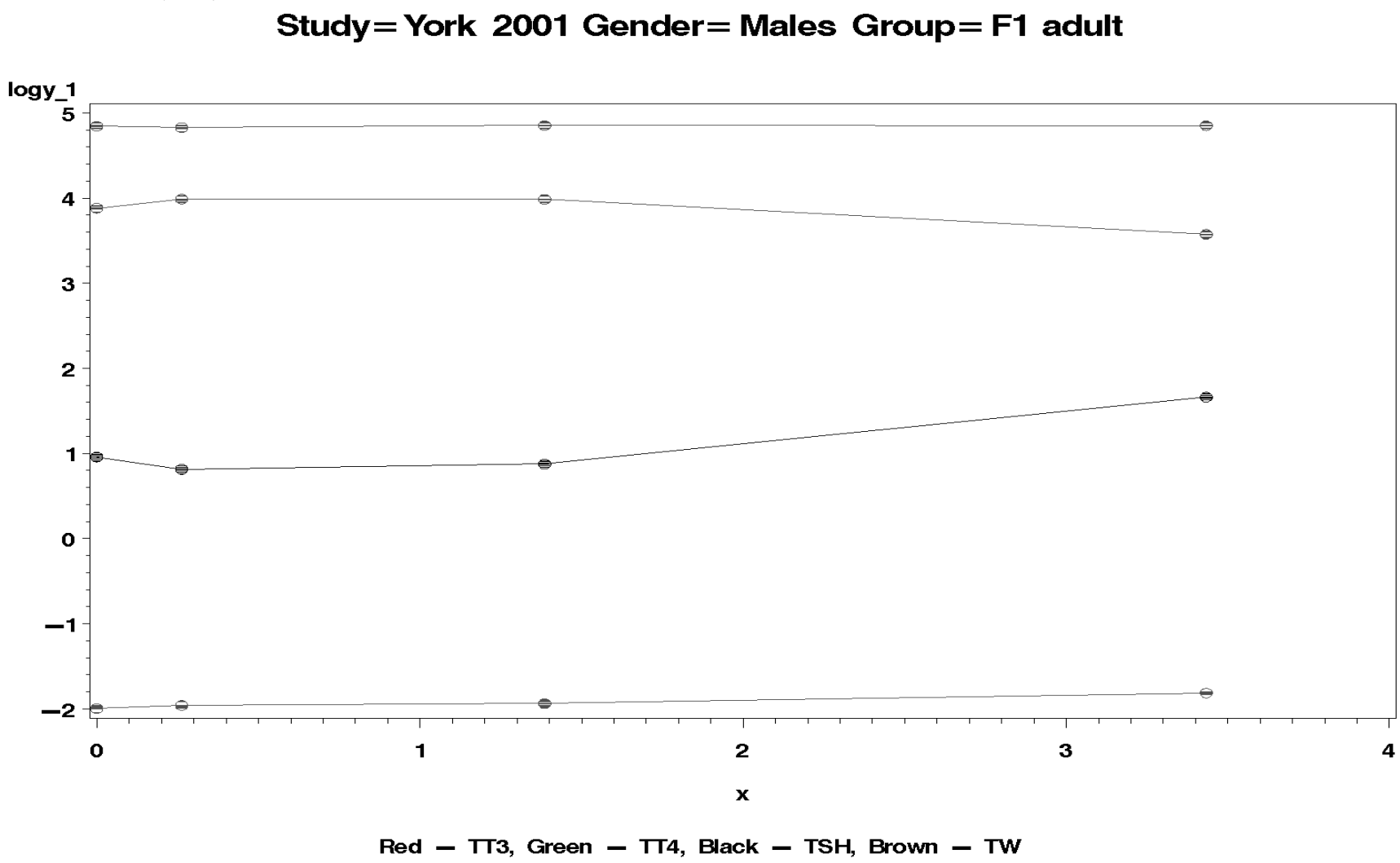
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4
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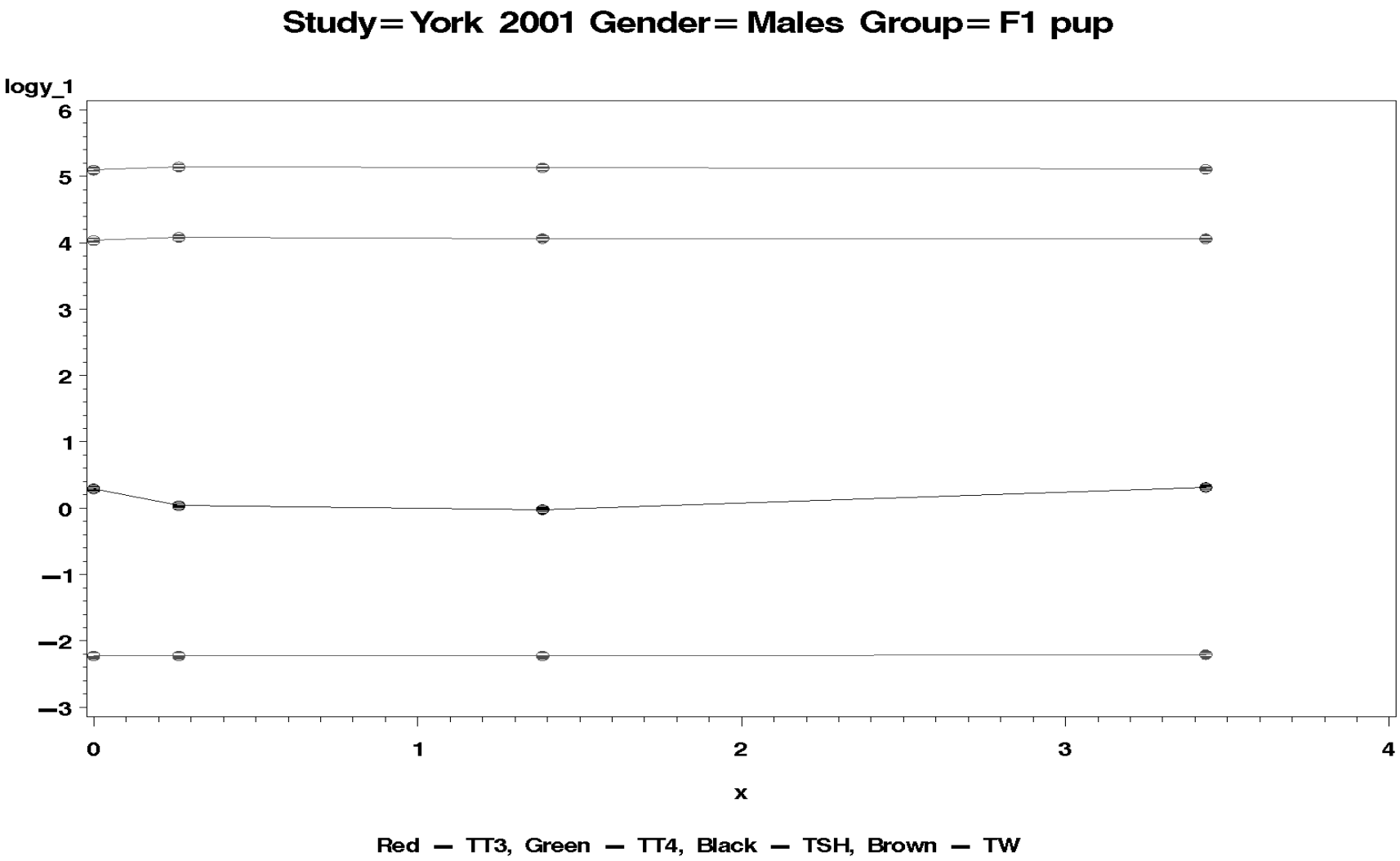
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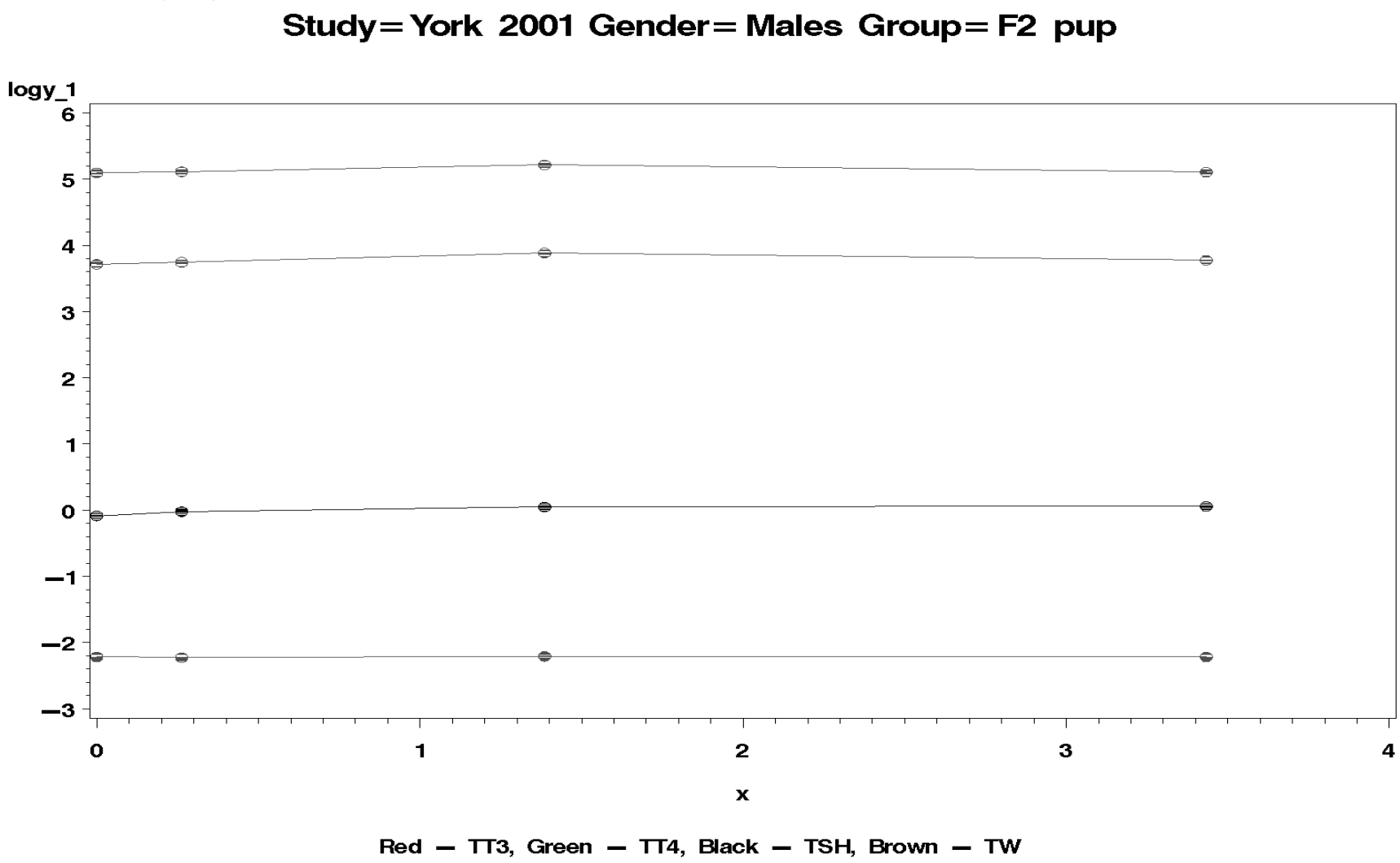
3
4
x
Red — TT3, Green — TT4, Black — TSH, Brown — TW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logy_1
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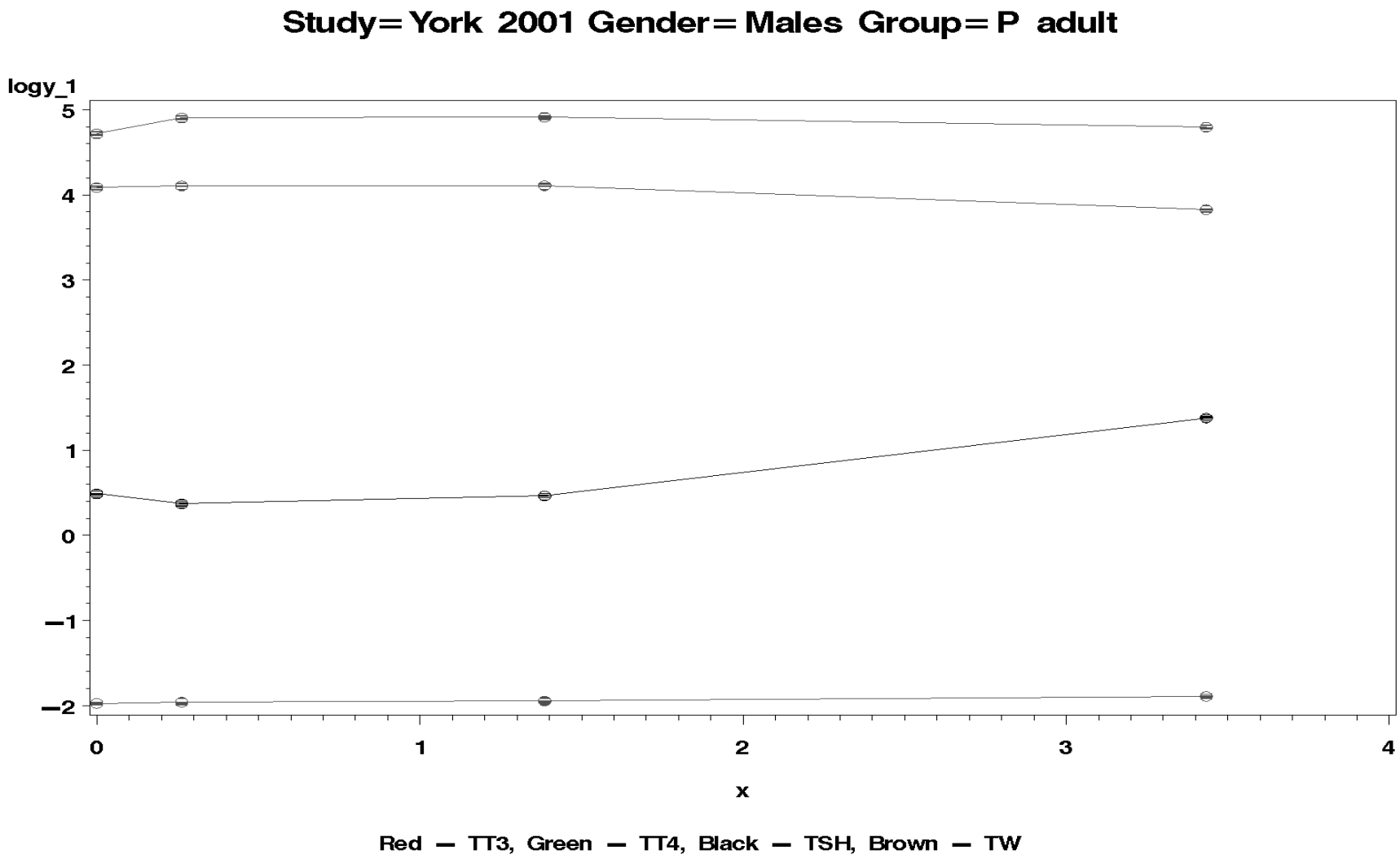
2
3
4
x
Red — TT3, Green — TT4, Black — TSH, Brown — TW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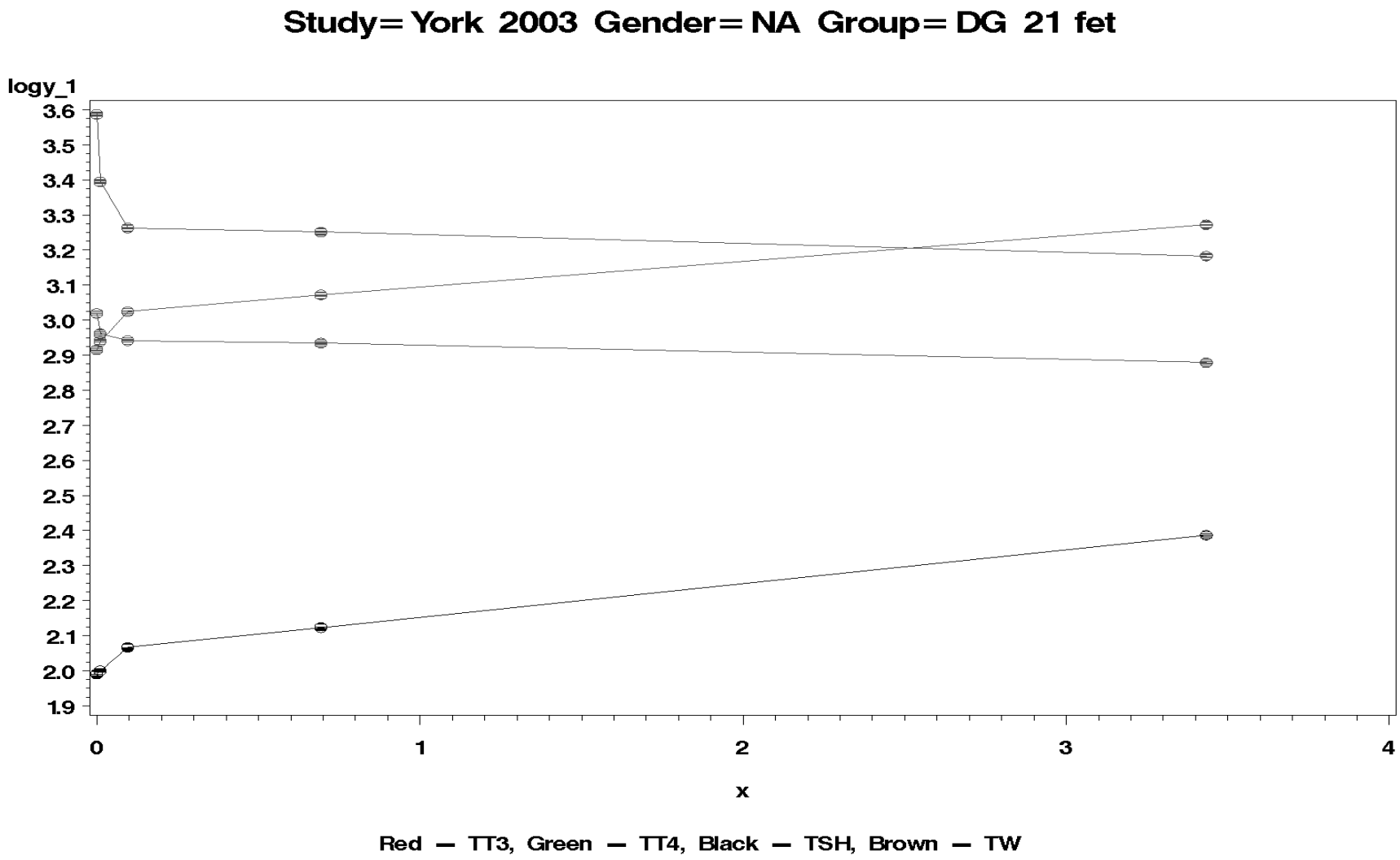
4
x
Red — TT3, Green — TT4, Black — TSH, Brown — TW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2.4
2.3
2.2
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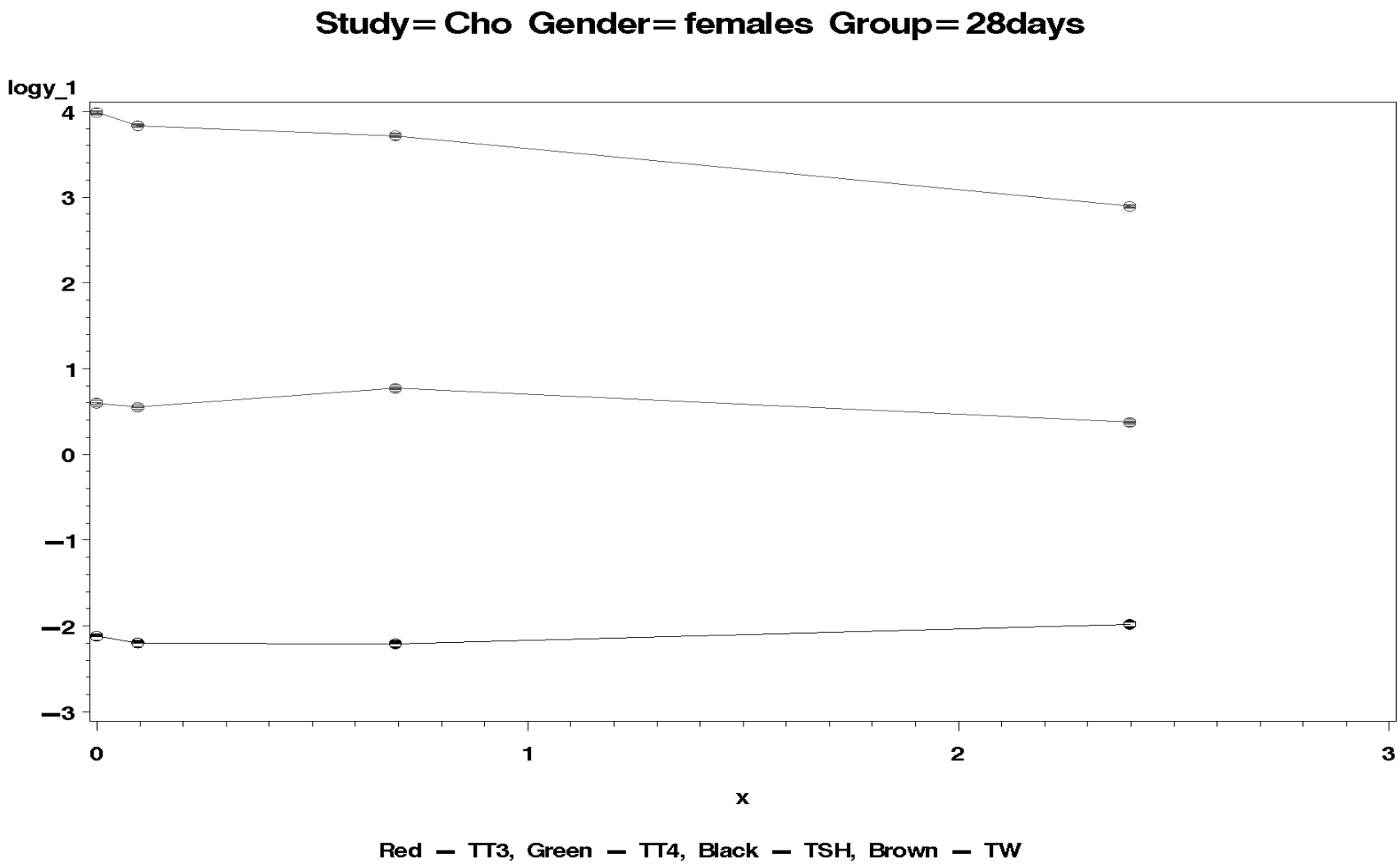
Red — TT3, Green — TT4, Black — TSH, Brown — TW

APPENDIX II

PTU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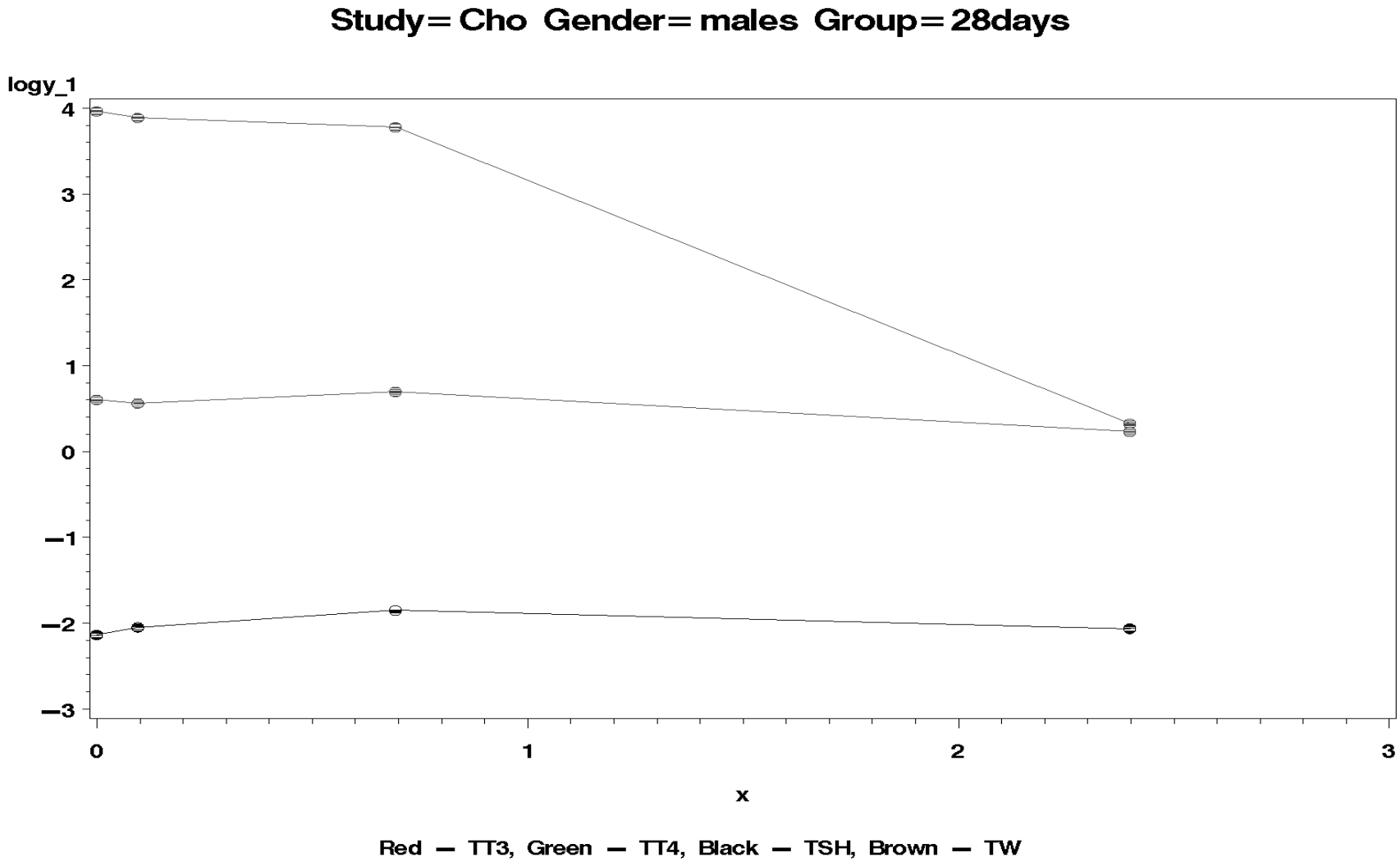
x
Red — TT3, Green — TT4, Black — TSH, Brown — TW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2
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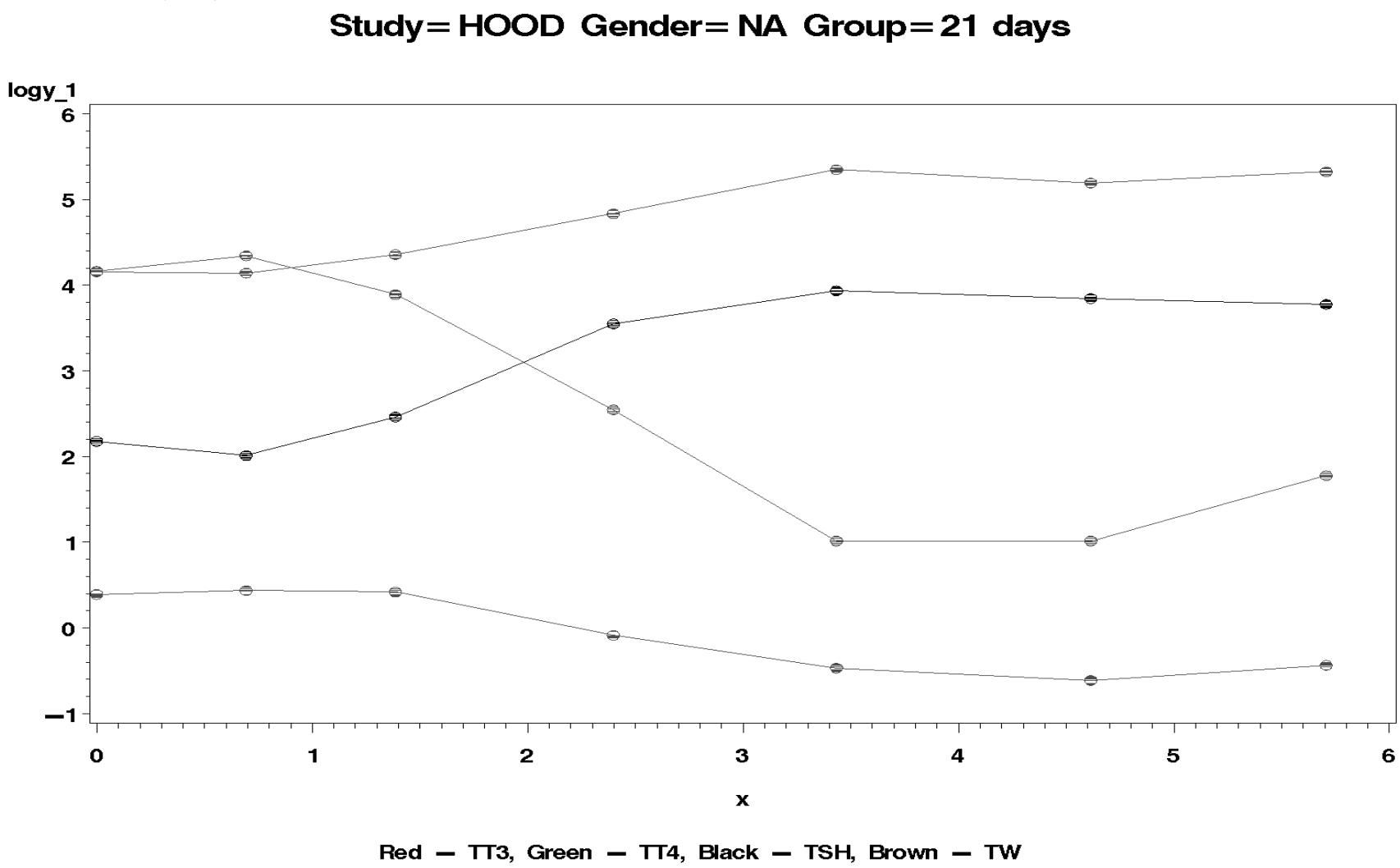
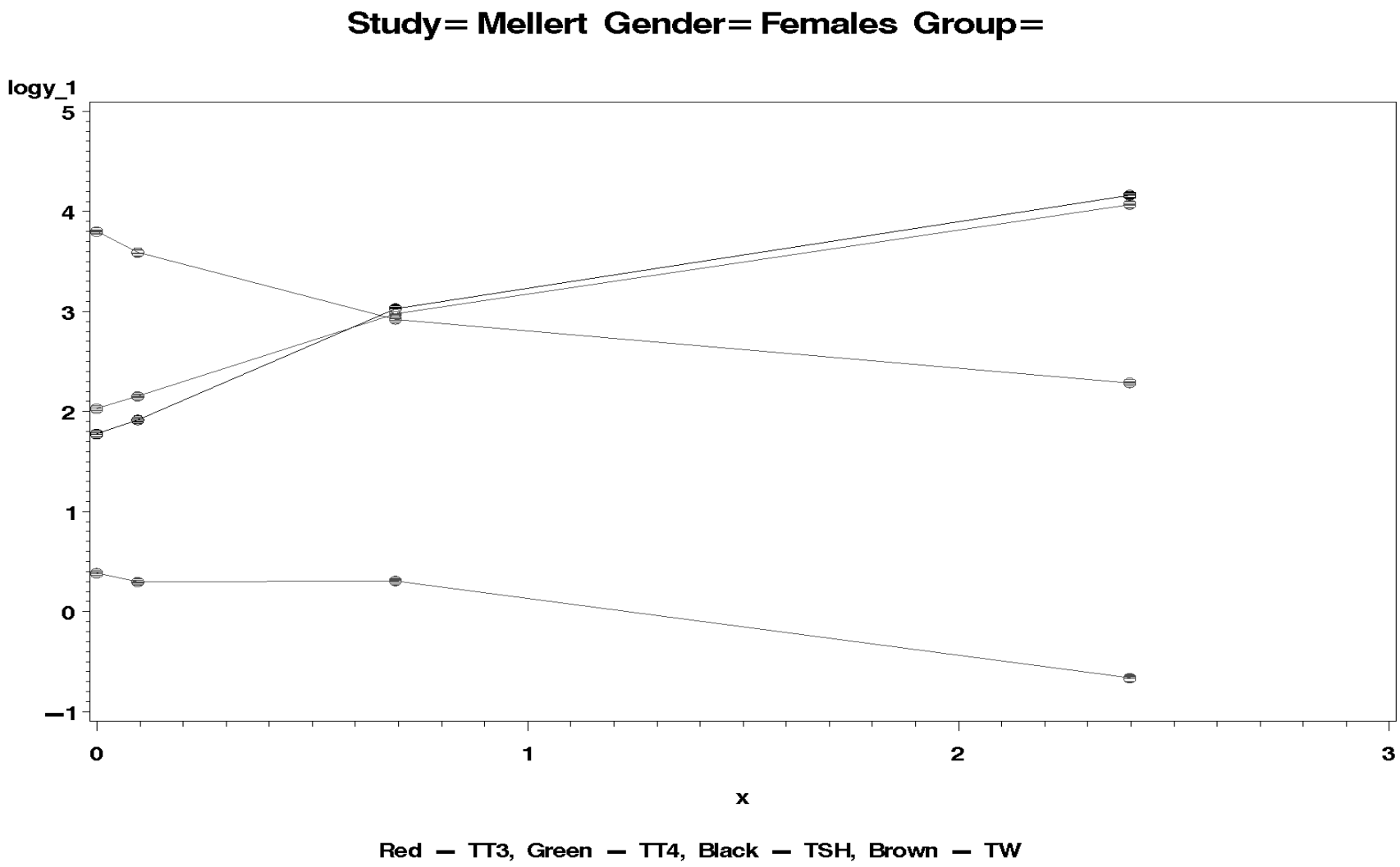
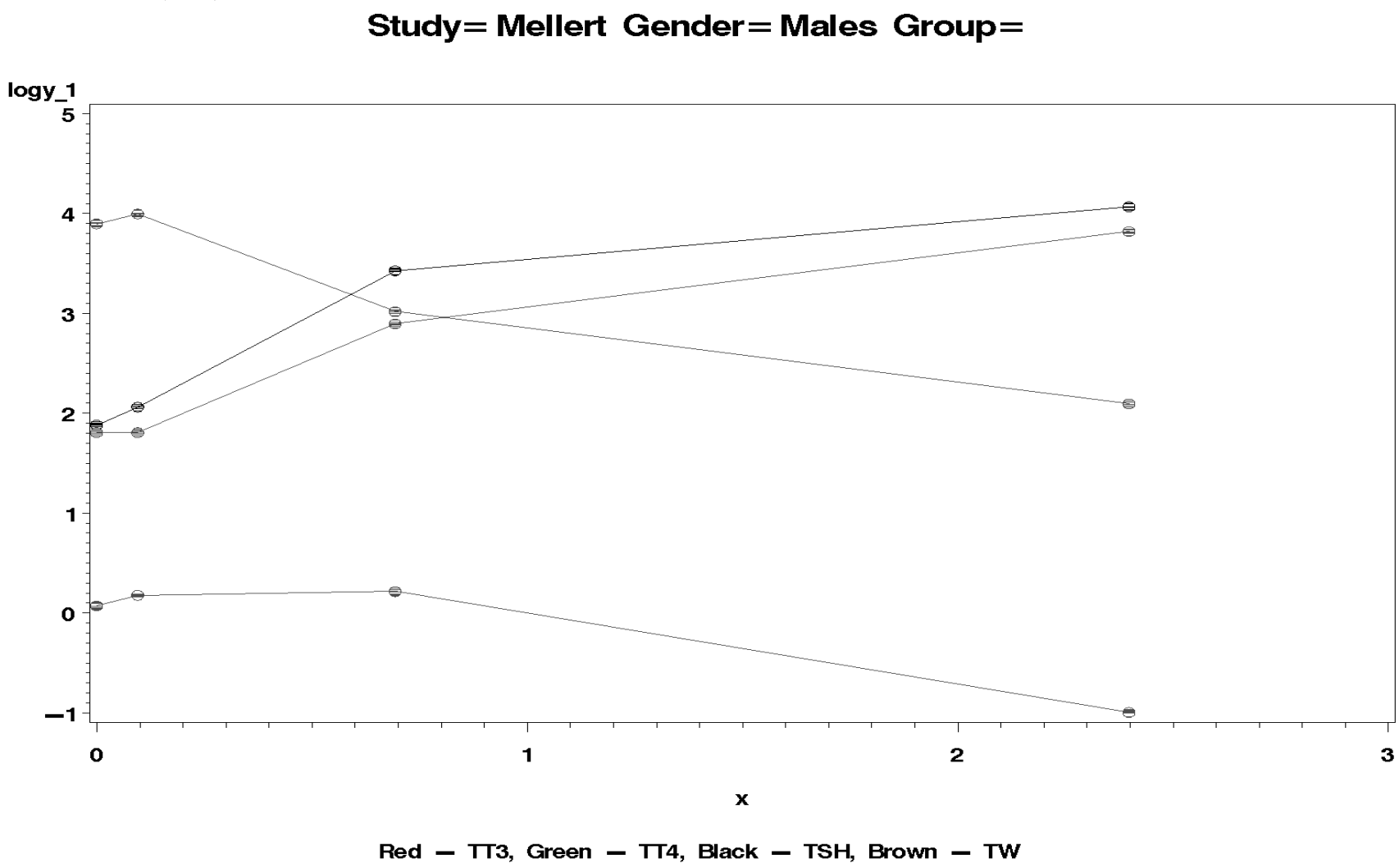
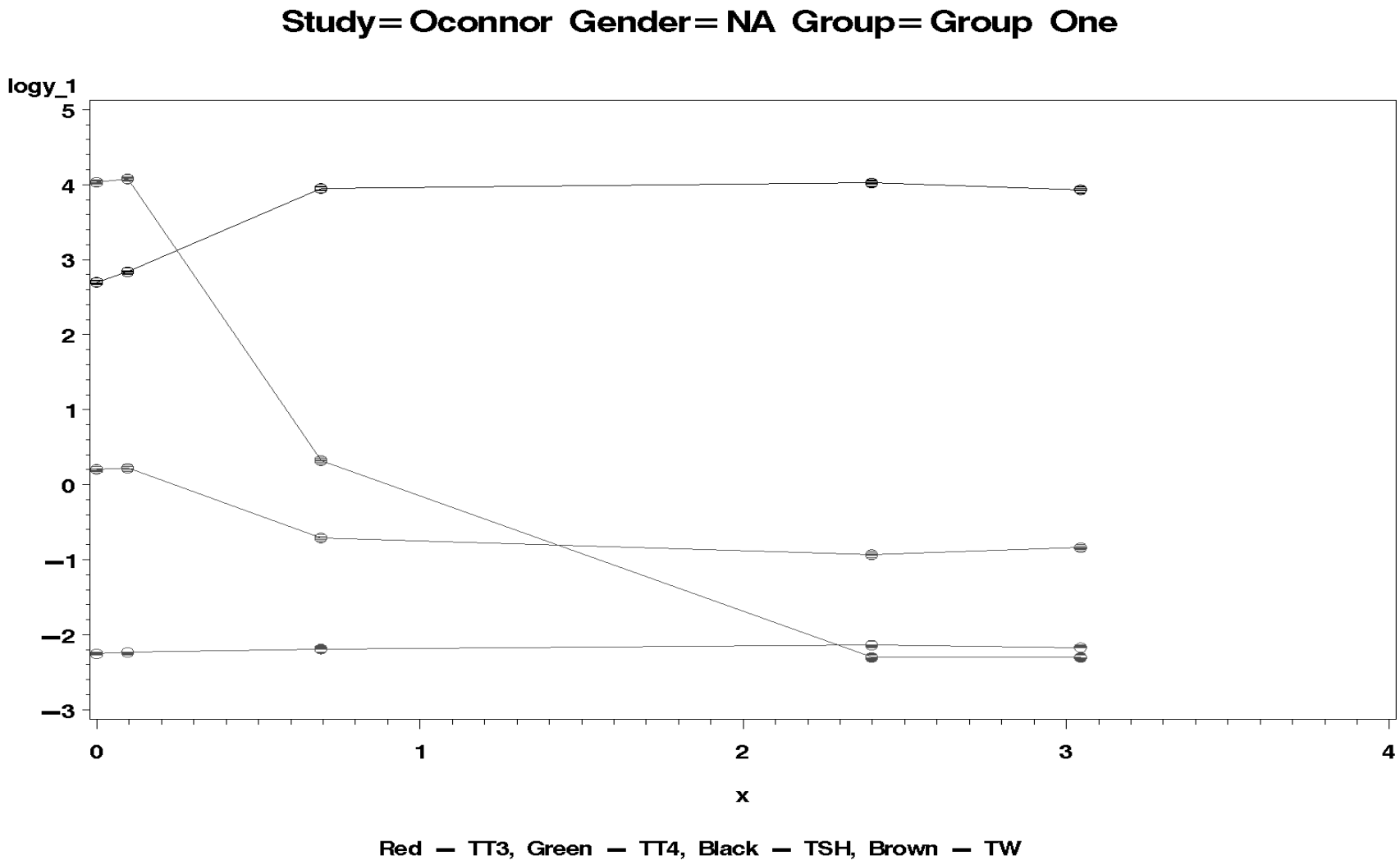
0
−1
−2
−3
0
1
2
3
x
Red — TT3, Green — TT4, Black — TSH, Brown — TW


Study= HOOD Gender= NA Group= 21 days
logy_1
6
5
4
3
2
1
0
−1
0
1
2
3
4
5
6
x
Red — TT3, Green — TT4, Black — TSH, Brown — TW

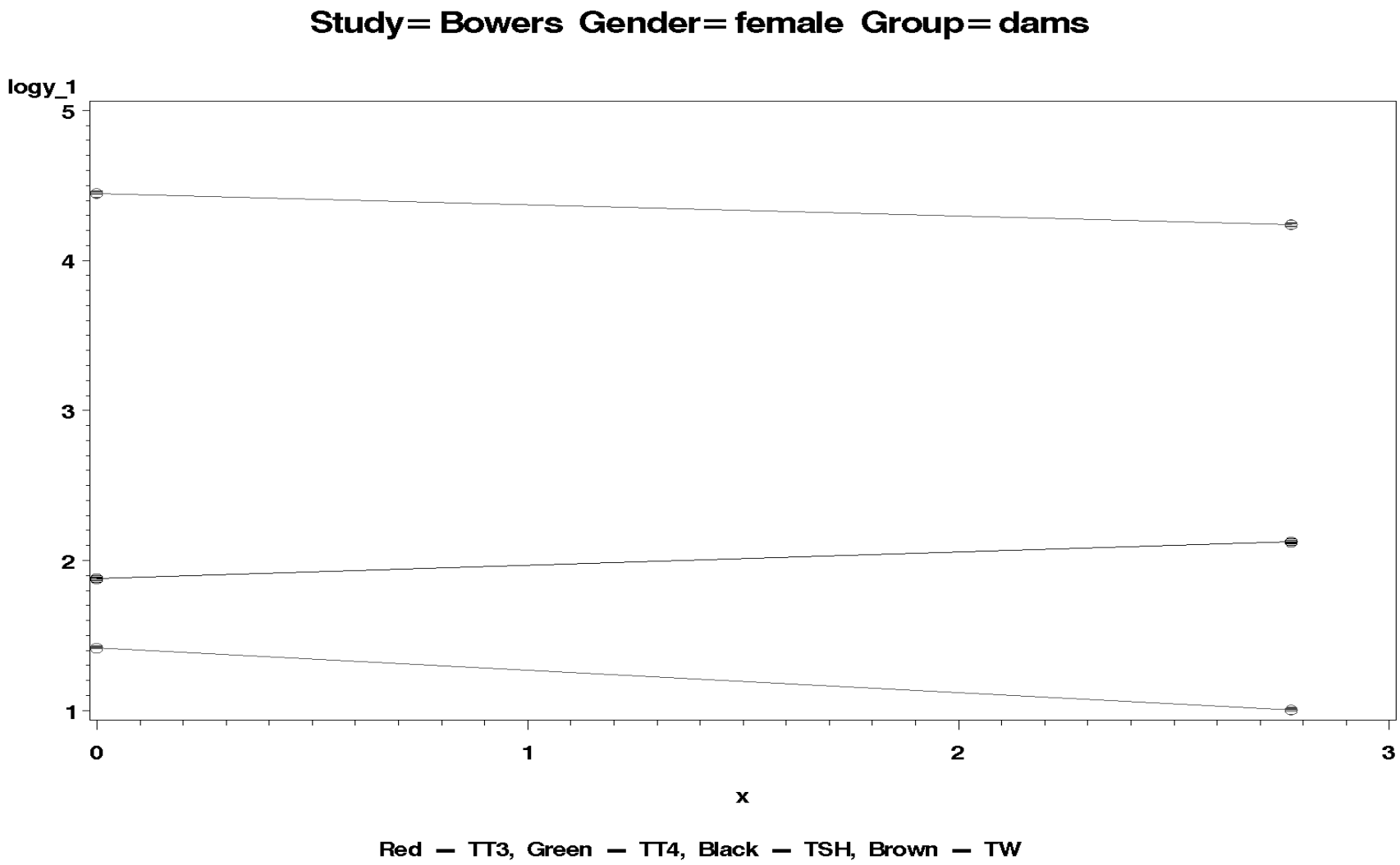

Study= Mellert Gender= Females Group=
logy_1
5
4
3
2
1
0
−1
0
1
2
3
x
Red — TT3, Green — TT4, Black — TSH, Brown — TW

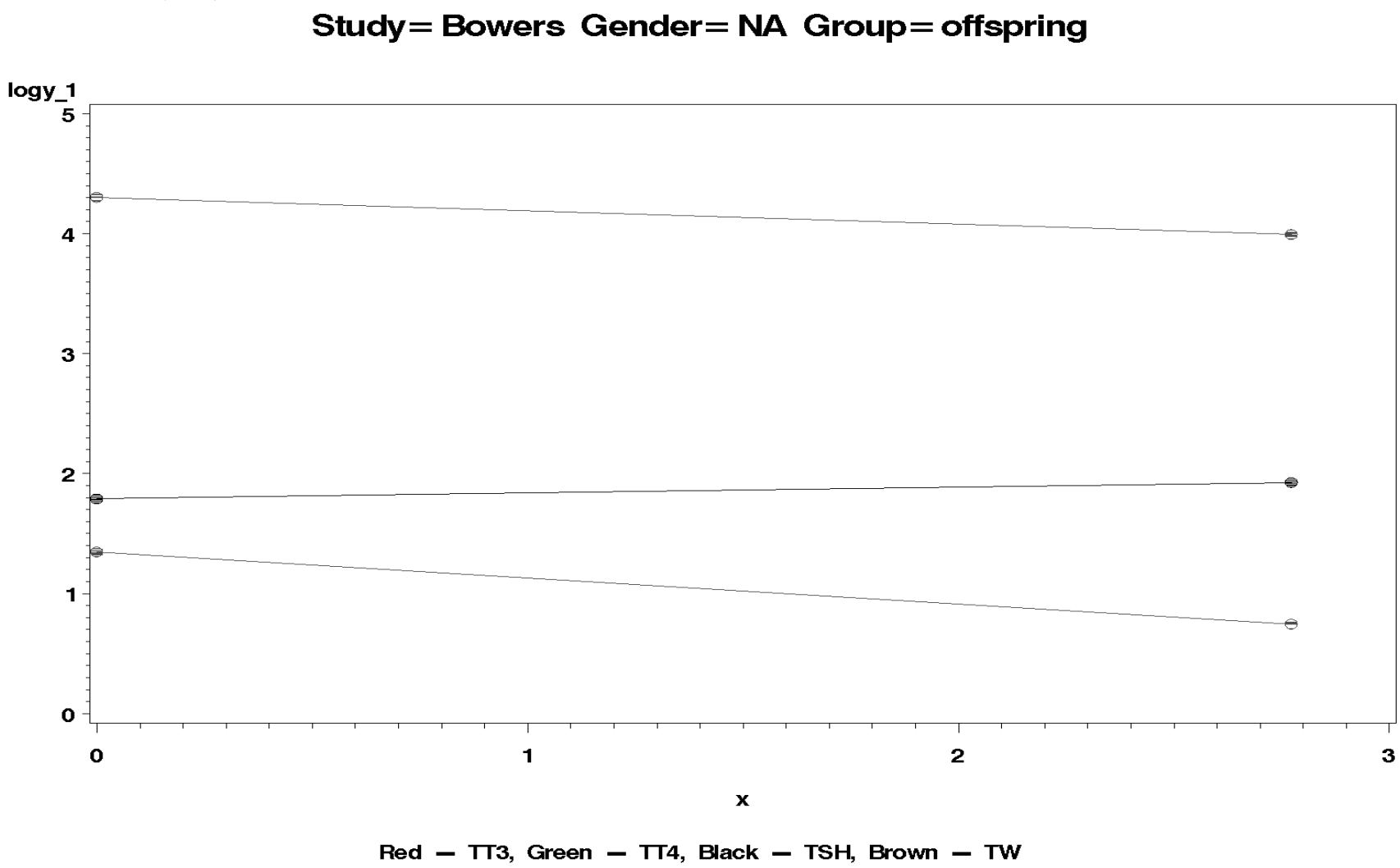

Study= Mellert Gender= Males Group=
logy_1
5
4
3
2
1
0
−1
0
1
2
3
x
Red — TT3, Green — TT4, Black — TSH, Brown — TW

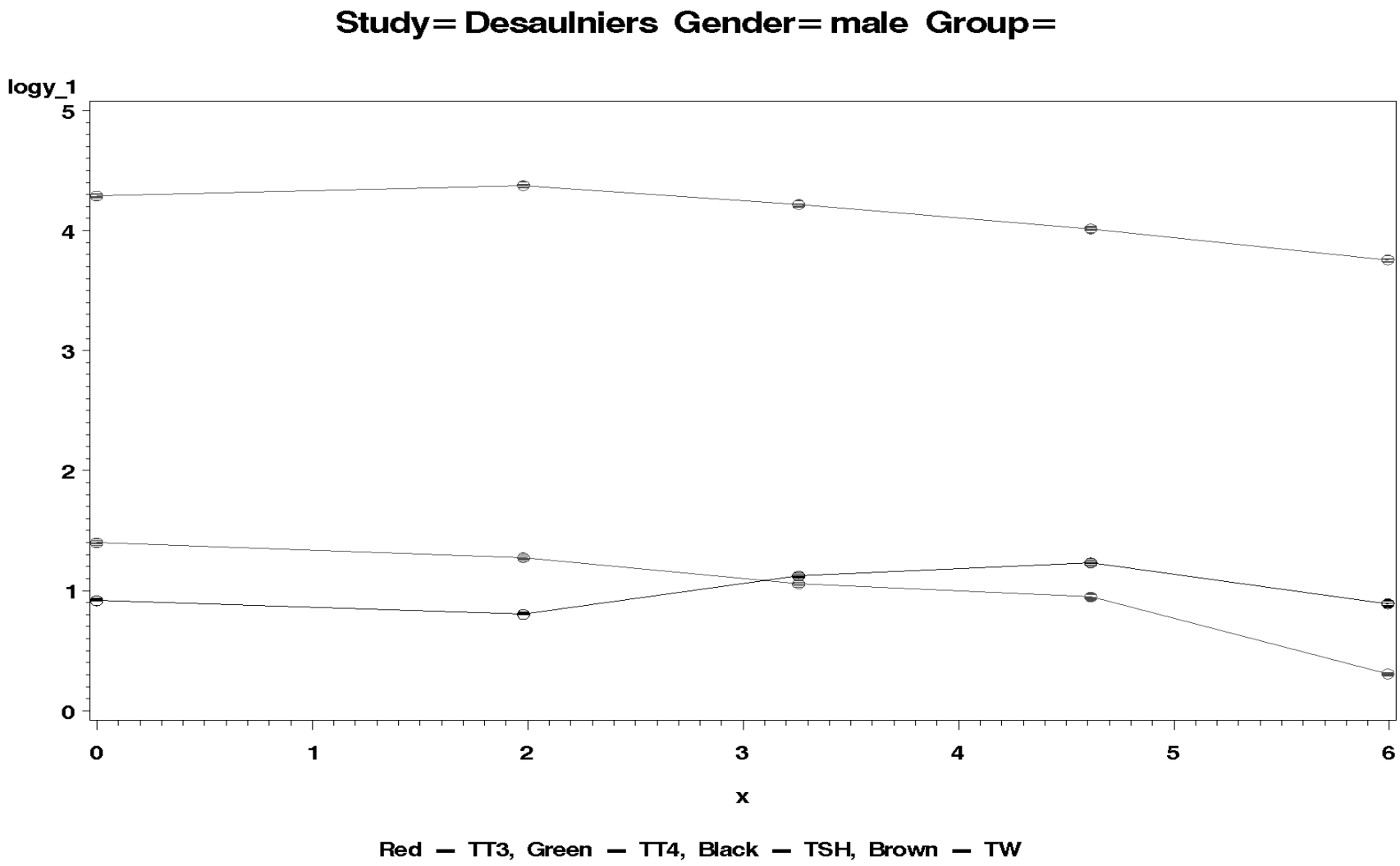

Study= Oconnor Gender= NA Group= Group One
logy_1
5
4
3
2
1
0
−1
−2
−3
0
1
2
3
4
x
Red — TT3, Green — TT4, Black — TSH, Brown — TW

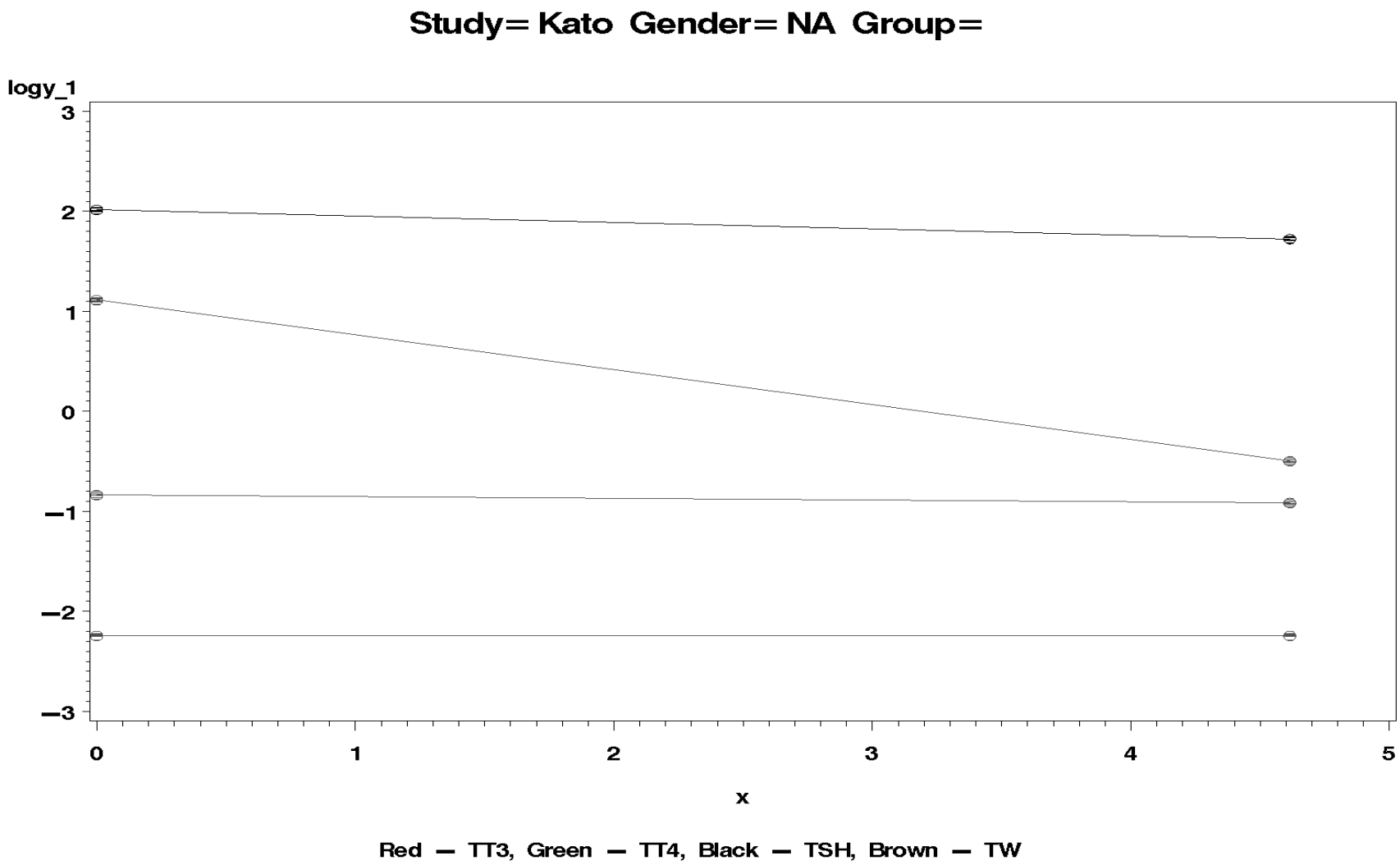
APPENDIX III

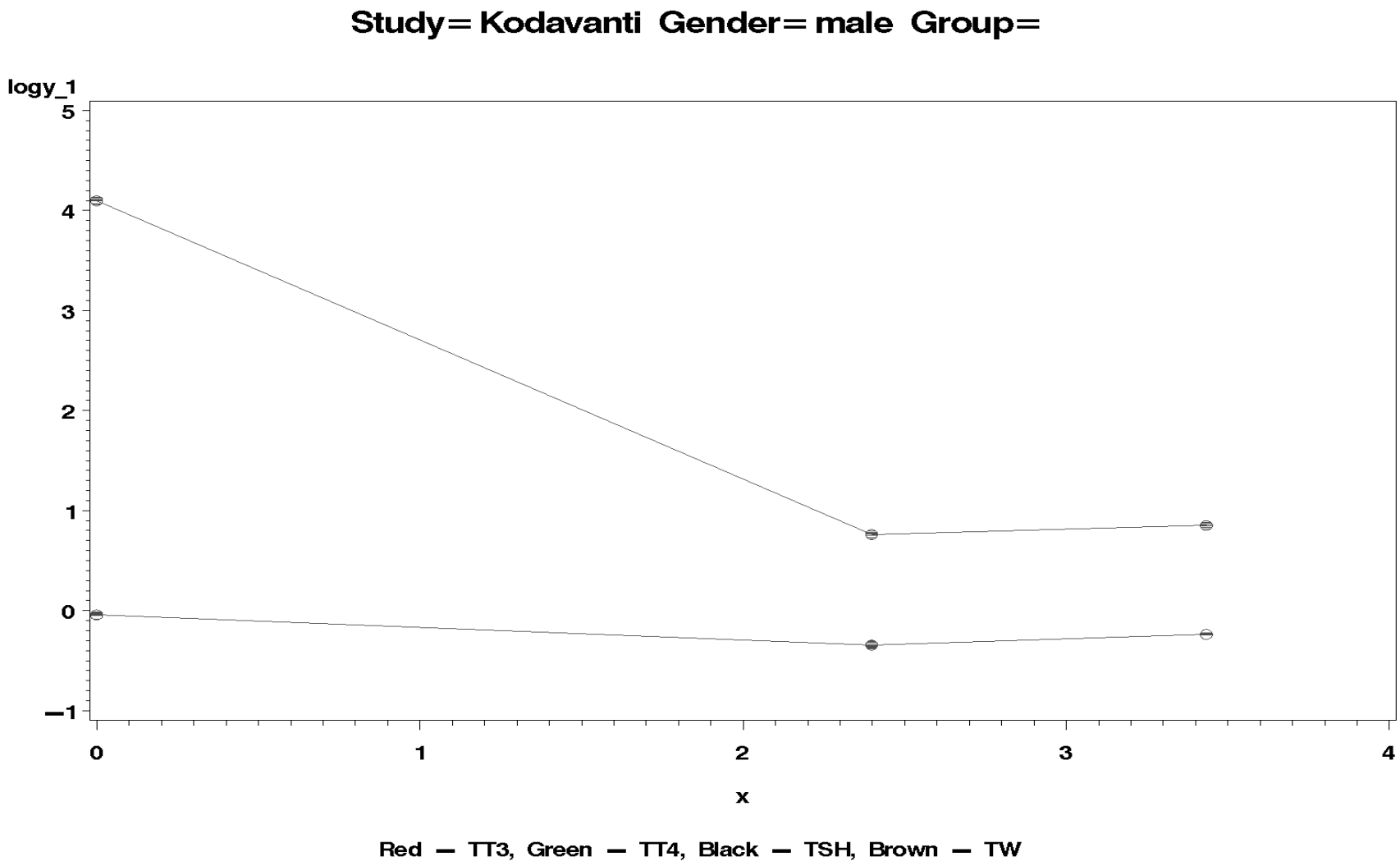
PCB

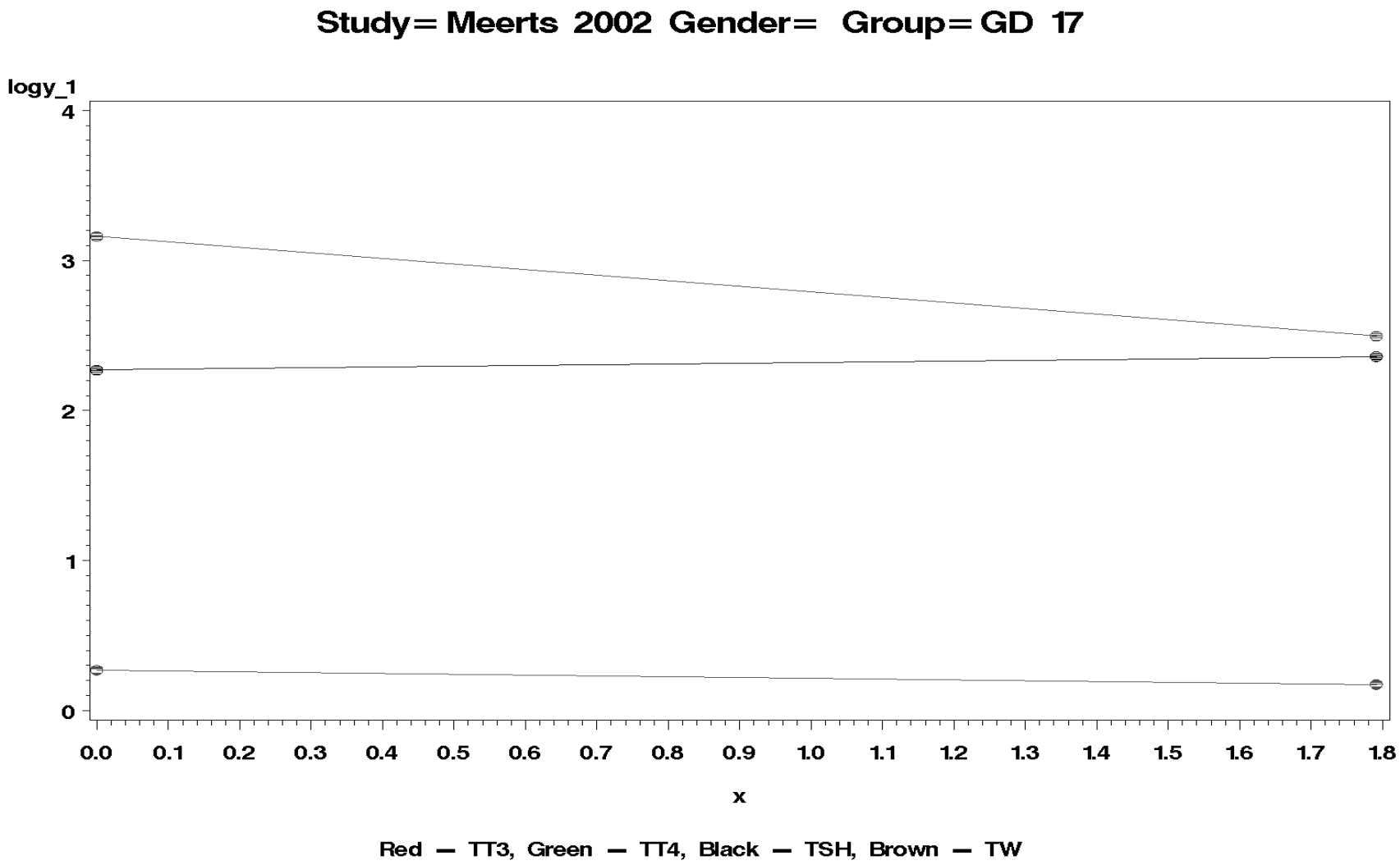

Study= Bowers Gender= female Group= dams
logy_1
5
4
3
2
1
0
1
2
3
x
Red — TT3, Green — TT4, Black — TSH, Brown — TW

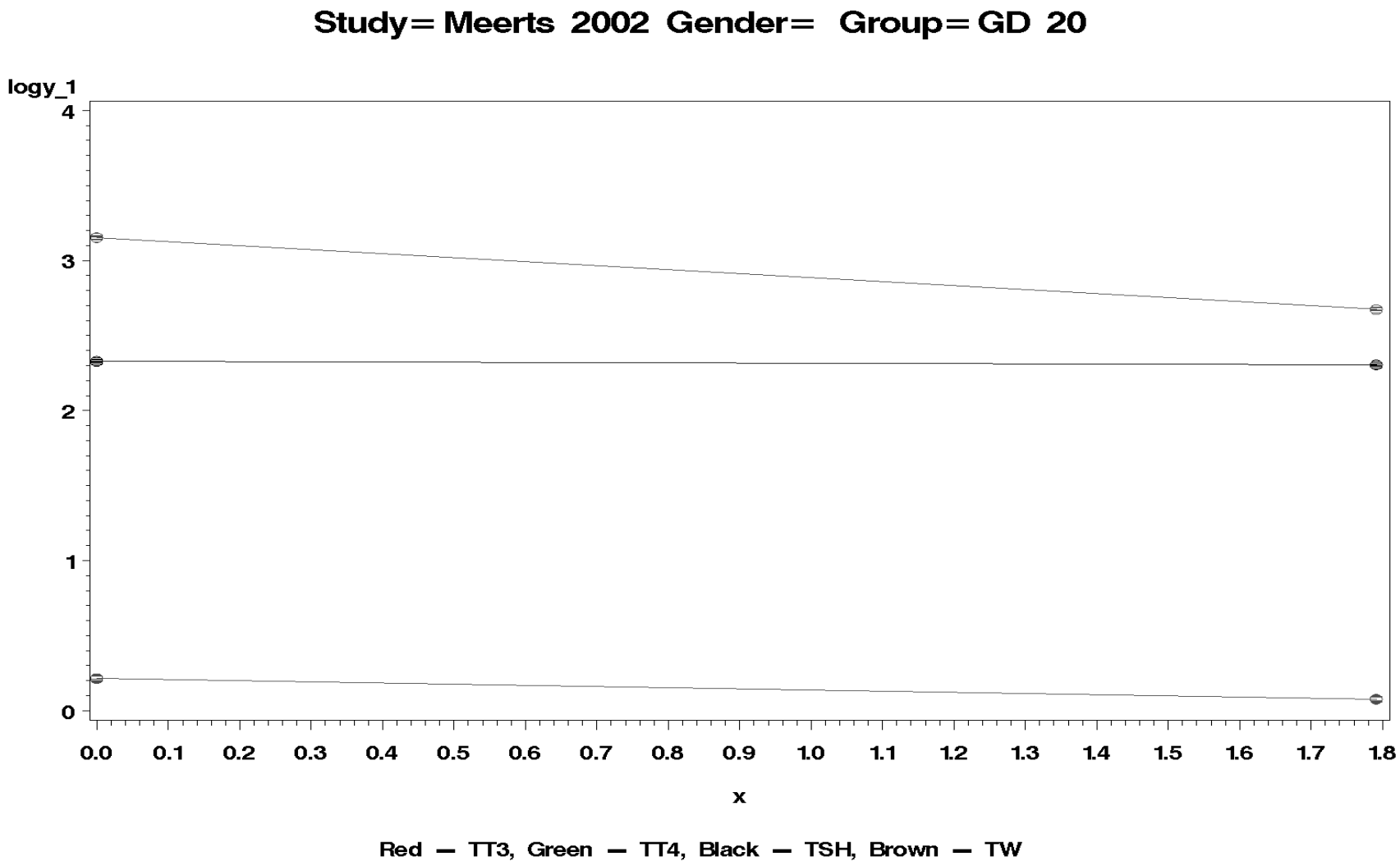

Study= Bowers Gender= NA Group= offspring
logy_1
5
4
3
2
1
0
0
1
2
3
x
Red — TT3, Green — TT4, Black — TSH, Brown — TW

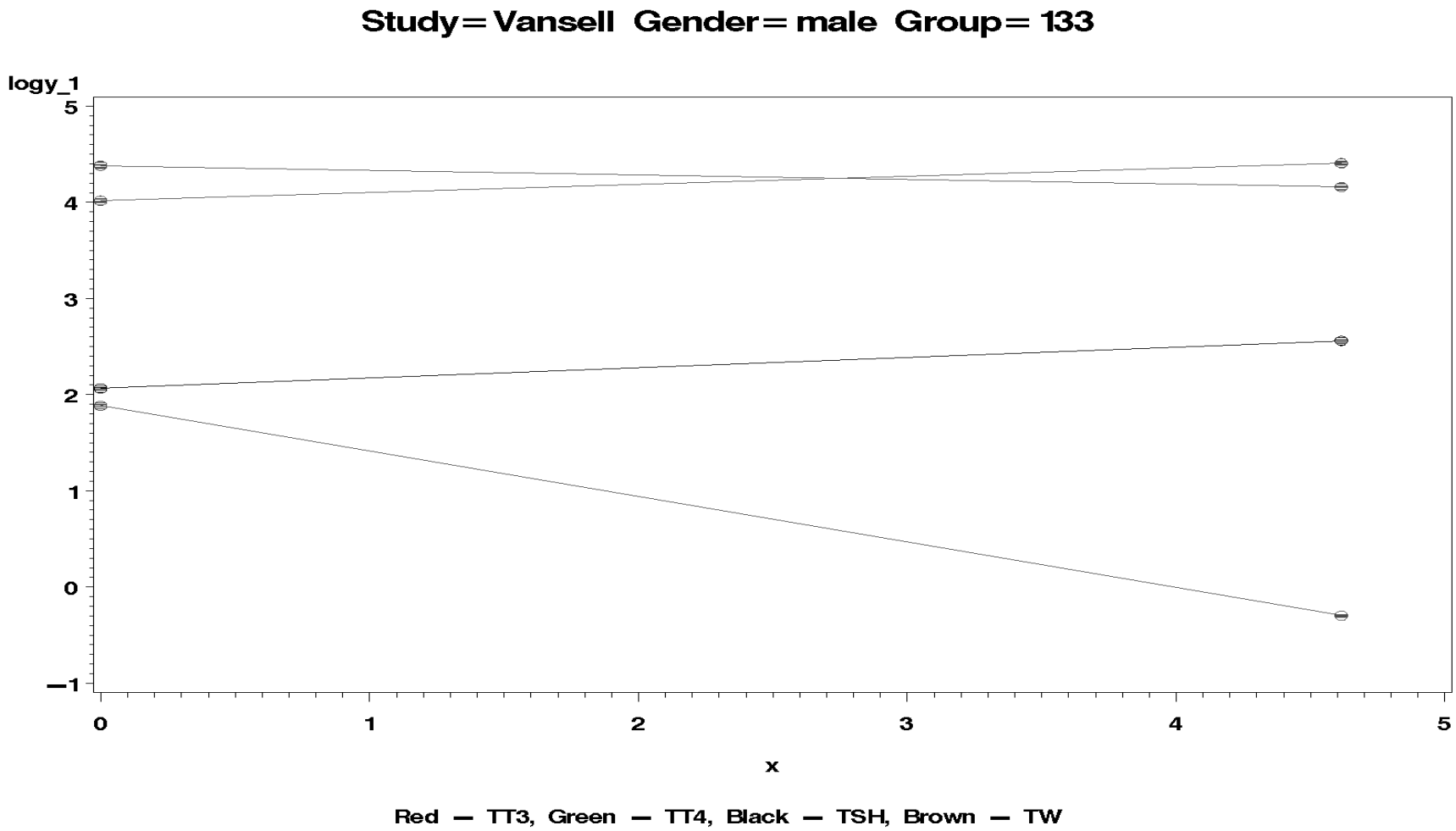

Study= Desaulniers Gender= male Group=
logy_1
5
4
3
2
1
0
0
1
2
3
4
5
6
x
Red — TT3, Green — TT4, Black — TSH, Brown — TW


Study= Kato Gender= NA Group=
logy_1
3
2
1
0
−1
−2
−3
0
1
2
3
4
5
x
Red — TT3, Green — TT4, Black — TSH, Brown — TW


Study= Kodavanti Gender= male Group=
logy_1
5
4
3
2
1
0
−1
0
1
2
3
4
x
Red — TT3, Green — TT4, Black — TSH, Brown — TW


Study= Meerts 2002 Gender= Group= GD 17
logy_1
4
3
2
1
0
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
1.1
1.2
1.3
1.4
1.5
1.6
1.7
1.8
x
Red — TT3, Green — TT4, Black — TSH, Brown — TW


Study= Meerts 2002 Gender= Group= GD 20
logy_1
4
3
2
1
0
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
1.1
1.2
1.3
1.4
1.5
1.6
1.7
1.8
x
Red — TT3, Green — TT4, Black — TSH, Brown — TW


Study=Vansell Gender=male Group=133
logy_1
5
4
3
2
1
0
−1
0
1
2
3
4
5
x
Red — TT3, Green — TT4, Black — TSH, Brown — TW