POLYCHLORINATED BIPHENYL EFFECTS ON AVIAN HEPATIC ENZYME INDUCTION AND THYROID FUNCTION

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Polychlorinated biphenyls (PCBs) decrease thyroid function in rats and mice by inducing activity of a liver enzyme, uridine diphosphate-glucuronosyltransferase (UDP-GT), thereby increasing thyroxine (T\(_4\)) clearance. This loss of T\(_4\) can lead to hypothyroidism. In this study, an assay was validated for measuring UDP-GT activity toward T\(_4\) in Japanese quail (\textit{Coturnix japonica}). Then UDP-GT induction by Aroclor 1254 was evaluated in quail, and quail and mice were compared in their responses to Aroclor 1254. In Experiment 1, Japanese quail and Balb/c mice were dosed orally with vehicle or Aroclor 1254 (250 or 500 mg/kg) and sacrificed five days later. In Experiment 2, Japanese quail were dosed orally with vehicle or Aroclor 1254 (500 mg/kg) and sacrificed either five or 21 days later. Total liver UDP-GT capacity increased with Aroclor 1254 exposure in all treatment groups of both species. Enzyme induction led to a trend to decreased plasma T\(_4\) concentrations at both doses and exposure times in quail and significantly decreased plasma T\(_4\) concentrations at both doses in mice. PCBs altered thyroid function in quail, but they did not become hypothyroid. This was in contrast to mice, which did become hypothyroid. It is unclear how PCBs affect the hypothalamic-pituitary-thyroid (HPT) axis in quail, and activation of the HPT axis appears to be inhibited in mice. Overall, quail showed a lesser response than mice to equivalent doses of Aroclor 1254, so it appears that birds may be less vulnerable to PCBs than mammals.
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Abbreviations

3MC................................................................. 3-methylcholanthrene
DDE.............................................................. dichlorodiphenyldichloroethylene
EROD.............................................................. ethoxyresorufin O-deethylase
HOM ................................................................... liver homogenate
HPT ................................................................. hypothalamic-pituitary-thyroid
ORD ................................................................. outer-ring deiodinase
PB................................................................... phenobarbital
PCB ................................................................. polychlorinated biphenyl
PCN ................................................................. pregnenolone-16α-carbonitrile
PHAH ................................................................ polyhalogenated aromatic hydrocarbon
pNP ................................................................. para-nitrophenol
RIA ................................................................. radioimmunoassay
T₃ ................................................................. triiodothyronine
T₄ ................................................................. tetraiodothyronine; thyroxine
T₄G ................................................................ T₄ glucuronide
TH ................................................................... thyroid hormone
TRH ................................................................. thyrotropin releasing hormone
TSH ................................................................. thyroid stimulating hormone; thyrotropin
UDPGA ........................................................... uridine diphospho-glucuronic acid
UDP-GT ............................................................. uridine diphosphate-glucuronosyltransferase
Chapter 1: Literature Review

Historical Significance of PCBs in the Great Lakes

Since the 1960s in some regions of the Great Lakes (USA), chemical contaminants, including polychlorinated biphenyls (PCBs), have had detrimental effects on at least 12 species of fish-eating wildlife, including nine bird species. These effects include population declines, thyroid defects, and reproductive abnormalities (review, Fox, 1993).

Decreases in thyroid function in these fish-eating birds of the Great Lakes have been suggested by thyroid gland hypertrophy (goiter) and developmental abnormalities that may be due to thyroid hormone (TH) deficiencies. Enlarged thyroid glands are indicative of activation of the hypothalamic-pituitary-thyroid (HPT) axis. All vertebrates require THs for normal development, so decreased development often indicates decreased thyroid function. Some of these developmental abnormalities include defects of the central nervous system and skeletal anomalies, such as incomplete skeletal ossification and stunted growth. For instance, embryos of fish-eating birds collected at contaminated sites in the Great Lakes had shorter femur and tarsal lengths than those collected at reference sites (reviews, Fox, 1993; McNabb and Fox, 2003; Scanes and McNabb, 2003).

Reproductive abnormalities in fish-eating birds also appear to have resulted from chemical contamination in the Great Lakes. These include altered sex ratios, abnormal breeding behavior, and decreased hatching success. For instance, it was found that female herring gulls (Larus argentatus) were nesting together and laying infertile eggs, probably due to a shortage of males. Also, adults were less attentive to their nests, therefore increasing the disappearance of eggs (review, Fox, 1993). Partially because of decreased attentiveness to nests, such as abandonment or inadequate incubation (review, Fox, 1993), but also because of other abnormalities, such as enlarged livers, there was high embryonic mortality (review, Fox, 1993), which could lead to population declines.

Many developmental abnormalities in fish-eating birds are thought to be due to some of the most common and persistent contaminants in the Great Lakes, polyhalogenated aromatic hydrocarbons (PHAHs), especially PCBs. These chemicals are lipophilic, so they are stored in body fats and biomagnify up the food chain, increasing exposure in top predators such as fish-eating birds. In addition, female birds deposit these chemicals in their eggs, exposing their young as embryos, when they may be most vulnerable to toxicants (reviews, McNabb and Fox, 2003; Scanes and McNabb, 2003).

Since these problems in wildlife of the Great Lakes were discovered in the 1960s, the concentrations of PCBs have decreased, with the greatest decreases occurring prior to 1985. The decreases in these chemicals have been associated with a reduction of developmental defects and embryonic mortality. However, due to the persistence of many of these contaminants and thus sustained lower exposure to them, these problems, including endocrine disruption, have not disappeared (Fox et al., 1998; review, Scanes and McNabb, 2003). PCBs and disruption of thyroid function will be discussed further in the following sections.
Polychlorinated Biphenyls

PCBs are some of the most abundant chemical contaminants in the Great Lakes. Their many uses, such as in dielectric fluids for transformers and capacitors, hydraulic fluids, paints, inks, plastics, and a number of other products, were due to their chemical stability and electrical insulating properties. Many types of PCB mixtures were produced; those with the trade name Aroclor were the most common (Erickson, 1997). Aroclors are designated by 4-digit numbers; the first two digits (usually a 12) represent the 12 carbons of the biphenyl, the structural “core” of all PCBs (Environment Canada, 2003). The last two numbers, with one exception (Aroclor 1016), represent the percent by molecular weight of chlorine in the mixture. PCBs were first regulated in 1976 by Congress with the Toxic Substances Control Act; in 1978, however, Congress banned the production and manufacture of PCBs (Erickson, 1997).

PCBs are composed of two aromatic rings (the biphenyl) with 1-10 chlorines attached (Fig. 1). The chemical formula for all PCBs is C_{12}H_{(10-n)}Cl_{n}, where \( n \) = any number from 1 to 10. There are 209 PCBs, referred to as congeners (Erickson, 1997). Most PCBs are non-coplanar, but there are 68 coplanar congeners (i.e., the biphenyls are on the same plane), 12 of which are dioxin-like (Environmental Protection Agency, 2006). Some of the dioxin-like PCBs, such as PCBs 77, 126, and 169, have been indicated in the general literature to be the most toxic congeners. The structure of PCBs leads to high chemical and physical stability, contributing to their persistence in the environment; however, the rate and mechanism of degradation differs depending on environmental conditions and on the specific structure of the PCB. The non-polar structure of PCBs makes them lipophilic, so they accumulate in body fats and can be biomagnified up the food chain (Erickson, 1997); this biomagnification causes top predators, such as fish-eating birds, to have the highest exposure to the toxic effects of PCBs. Herring gulls are a common example of predators exposed to a high level of PCBs, which makes them a sentinel species to study for effects of PCBs on birds.

Thyroid Function

Structure. In birds, the thyroid gland consists of two separate glands, located ventrolaterally in the neck on each side of the trachea. Thyroid glands consist of many follicles, which are spheres of one layer of epithelial cells, with the lumen filled with colloid. Colloid is composed of the protein thyroglobulin, within which THs are stored. This is a form of extracellular storage of THs, which is unique to the thyroid gland. Because of this storage, large quantities of THs may be stored for long periods of time (review, McNabb, 2000).

Function. THs have several functions. They play a critical role in growth, development, and differentiation (especially of muscle, the skeleton, and the central nervous system) in vertebrates, as well as in metabolism in birds and mammals, the two homeothermic classes of vertebrates (review, McNabb, 1992). It is because of the importance of THs in development and metabolism that any type of disruption of thyroid function can be potentially damaging to individuals or to populations.
**Thyroid hormone synthesis.** THs are synthesized by attaching iodine to tyrosine residues that are within the protein thyroglobulin. Thyroglobulin is made in the epithelial cells of the follicles, and iodine is transported into the cells from capillaries surrounding the follicles. Thyroglobulin and iodine move into the colloid via exocytosis, where THs are synthesized by thyroid peroxidase and stored within thyroglobulin (review, Taurog, 1996). When the THs are mobilized, thyroglobulin moves back into the epithelial cell via endocytosis and is cleaved by several enzymes (including cysteine proteinase I and cathepsin-B), producing THs. After the thyroglobulin has been cleaved, the THs leave the cell and enter the bloodstream. Thyroxine (T₄), which has 3, 5, 3',5'-iodination on thyronine (Fig. 2), makes up more than 90% of the TH produced by the thyroid gland. The rest is triiodothyronine (T₃), which has 3, 5, 3'-iodination on thyronine (Fig. 3; review, McNabb, 1992).

THs are transported in the blood by binding proteins. Some THs, however, are free (not bound to a binding protein; less than 1% in humans), enabling them to move out of capillaries and into cells, becoming biologically active. T₄ binds with about 10 times greater affinity than T₃ to binding proteins, which makes T₃ better able to dissociate from binding proteins and become available for transport into cells (review, Robbins, 1996).

In contrast to T₄, which is produced primarily by the thyroid gland, most T₃, the biologically active TH, is produced in extrathyroidal tissues by conversion from T₄. This conversion is catalyzed by the enzyme 5'-deiodinase, which produces T₃ by removing the iodide in the 5' position of T₄ (review, McNabb, 1992).

**Regulation of thyroid function.** Thyroid function is regulated by the HPT axis (Fig. 4), which is a homeostatic mechanism for maintaining circulating TH concentrations. In response to low circulating THs, the hypothalamus produces thyrotropin releasing hormone (TRH), which stimulates the anterior pituitary gland. The anterior pituitary then releases thyroid stimulating hormone (TSH or thyrotropin), which in turn stimulates all aspects of thyroid gland function (iodine uptake, TH production, TH release, and growth of the thyroid gland). This system regulates thyroid function by detecting a deficiency or abundance of THs. If the circulating TH concentration is too high, then this will have a negative feedback effect on production of TRH and TSH by the hypothalamus and anterior pituitary, respectively (i.e., function of the hypothalamus and pituitary will decrease in response to increased circulating TH concentrations). A decrease in TSH production will lead to a decrease in thyroid gland function, including TH production. If the concentration of circulating THs is low, then the hypothalamus and anterior pituitary will produce more TRH and TSH, respectively, causing the thyroid gland to produce more THs (review, Scanlon and Toft, 1996).

**Environmental Contaminant Effects on Thyroids**

Hyperplasia and microfollicular goiters were common in Great Lakes herring gulls (*Larus argentatus*) sampled between 1974 and 1983. The reference site used was the Bay of Fundy, which has a higher iodine concentration than the Great Lakes, therefore possibly affecting the results of this study. However, the differences in iodine concentrations among lakes did not correspond to differences in thyroid pathology, suggesting that differing iodine concentrations would not affect the results of this study.
(Moccia et al., 1986). From 1974-1993, especially before 1985, in several herring gull colonies of the Great Lakes, PCBs, dieldrin, mirex, and DDE concentrations decreased in livers. By the end of this period, in the early 1990s, the severity of goiter in Great Lakes birds had decreased, corresponding to the decreased concentrations of organochlorines (including PCBs; Fox et al., 1998).

**Biotransformation Enzymes**

Biotransformation, divided into Phase I and II reactions, depending on the type of reaction, is the metabolism of chemicals, including environmental contaminants. Biotransformation alters their biological activity and allows them to be excreted more easily, either in the feces (after being secreted into bile) or in the urine. Even if a conjugated chemical is secreted into bile, it may be hydrolyzed by gastrointestinal enzymes, which might free the contaminant from the conjugate; this often allows it to be reabsorbed into circulation. Environmental contaminants often induce activity of these enzymes, causing increased biotransformation of the contaminant as well as other substrates such as THs, thus increasing their clearance. Biotransformation enzymes are present in many tissues, but predominantly in the liver. Most metabolism of environmental contaminants occurs in hepatic parenchymal cells, and within these cells, most of the metabolizing enzymes are found in the endoplasmic reticulum (Franklin and Yost, 2000).

**Phase I and II biotransformations.** Although the ultimate result of biotransformation (Phases I and II) is to enhance excretion of a chemical, Phase I reactions may yield products with enhanced activity. Oxidations, hydrolyses, and reductions are the most important Phase I biotransformations. One of the most common enzymes that catalyzes Phase I reactions is cytochrome P450, which is found in the liver and is responsible for many oxidations (Franklin and Yost, 2000).

Generally, products of Phase II reactions are not biologically active and are more water soluble than before the reaction. The most common Phase II reaction in mammals is glucuronidation; however, sulfation (the primary method of T₃ conjugation; Leonard and Koehrle, 2000) and glutathione conjugations also are important (Franklin and Yost, 2000).

A chemical may undergo one phase without the other, or it may undergo both phases. Phase I usually precedes Phase II, but it is possible for Phase II to occur first (Franklin and Yost, 2000).

**Uridine diphosphate-glucuronosyltransferase (UDP-GT).** Glucuronidations are catalyzed by UDP-GT, a Phase II biotransformation enzyme, which involves the transfer of a glucuronic acid from a uridine diphospho-glucuronic acid (UDPGA) cofactor (formed from carbohydrates in the liver) to a carboxyl, hydroxyl, or amine group on the substrate. Glucuronidation increases the substrate’s solubility in water, therefore enhancing its ability to be excreted in the feces or the urine (Franklin and Yost, 2000).

There are several isozymes of UDP-GT. In rats, 16 isozymes have been identified, and 19 forms have been identified in humans (Vansell and Klaassen, 2002); there is no information on different UDP-GT isozymes in birds. UDP-GT acts on a wide range of substrates, but many substrates are glucuronidated only by certain isozymes.
One commonly used substrate, *para*-nitrophenol (*p*NP), is glucuronidated by almost all isozymes (Franklin and Yost, 2000). *T*₄ is a substrate only known to be glucuronidated by two of the isozymes in rats, and *T*₃ is glucuronidated by at least one isozyme in rats (Vansell and Klaassen, 2002).

Glucuronidation of *T*₄ occurs mainly in the liver, but it also may occur at other sites such as the kidney and intestine (review, Curran and DeGroot, 1991). In one study on rats, it was found that enhanced *T*₄ UDP-GT activity was associated with increased transcription of two isozymes, UGT1A1 and UGT1A6, suggesting that these isozymes act on *T*₄ (Vansell and Klaassen, 2002). Some xenobiotics are capable of inducing different isozymes (Franklin and Yost, 2000), including UGT1A1 and UGT1A6. Induction of these two enzymes, as well as any others that might glucuronidate *T*₄, can cause a decrease in the concentration of circulating *T*₄. Consequences of UDP-GT induction toward *T*₄ will be discussed in the next sections.

**UDP-GT Induction and Thyroid Function**

There is evidence that different UDP-GT isozymes glucuronidate *T*₄, based on studies using different types of enzyme inducers. UDP-GT activity with *T*₄ as substrate was increased by exposure to pregnenolone-16α-carbonitrile (PCN), phenobarbital (PB), and 3-methylcholanthrene (3MC) in mice (Hood et al., 2003) and rats (Barter and Klaassen, 1992b; Barter and Klaassen, 1994; Hood and Klaassen, 2000; Liu et al., 1995) and by several other microsomal enzyme inducers in rats, each of which induces a different isozyme of UDP-GT. This indicates that several UDP-GT isozymes can glucuronidate *T*₄ (Barter and Klaassen, 1992a). Biliary excretion of *T*₄ glucuronide (*T*₄G) was increased by PCN and 3MC (Vansell and Klaassen, 2001), indicating that the isozymes induced by these chemicals are ones that glucuronidate *T*₄. PCN, PB, and 3MC have been shown to decrease circulating *T*₄ concentrations in rats (Barter and Klaassen, 1992b; Barter and Klaassen, 1994; Liu et al., 1995). The rats used in these studies were thyroidectomized and were maintained at euthyroid concentrations by TH pumps. This indicates that TH concentrations were reduced at least in part by an extrathyroidal mechanism, which supports the hypothesis that UDP-GT induction contributes to reduced circulating *T*₄ concentrations. In addition, PCN (Barter and Klaassen, 1994; Hood and Klaassen, 2000; Liu et al., 1995; Vansell and Klaassen, 2001), PB (Barter and Klaassen, 1994; Hood and Klaassen, 2000; Liu et al., 1995), and 3MC (Barter and Klaassen, 1994) have been shown to increase circulating TSH in rats, which is a physiological response to low TH concentrations.

**PCB Effects on UDP-GT Induction and Thyroid Function**

Several PCB mixtures and a few individual congeners have been studied for their effects on UDP-GT induction. Almost all of these studies have been done on rats or mice, and only a very few have been done on birds.
**Mammals**

**Aroclor 1254.** Aroclor 1254 was a commonly used PCB mixture and has been studied for its effects on T4 concentrations, UDP-GT activity, TSH stimulation, and T3 concentrations in rats and mice. UDP-GT activity toward T4 has been increased consistently by Aroclor 1254 at the higher doses and longer exposure times tested. Glucuronidation of T4 was significantly increased in mice at 10 ppm Aroclor 1254 for 21 days (Hood et al., 2003), was significantly increased in rats at 30 ppm (but not 10 ppm) Aroclor 1254 for 15 days (Liu et al., 1995), and was significantly increased in rats at 100 ppm (but not 50 ppm) Aroclor 1254 for 7 days (Hood and Klaassen, 2000). The differences in effects among the three studies may be due to the shorter lengths of time used when testing the higher doses. Two other studies showed that 250 ppm Aroclor 1254 significantly increased UDP-GT activity toward T4 in rats (Barter and Klaassen, 1992b; Barter and Klaassen, 1994).

Evidence of T4 glucuronidation has also been indicated by increased transcription of UDP-GT mRNA and by increased biliary excretion of T4G. Exposure to 100 ppm Aroclor 1254 for 7 days in rats significantly increased the transcription of mRNA of two UDP-GT isozymes (UGT1A6 and UGT1A7), but did not increase transcription of any other UDP-GT mRNAs. UGT1A6 is known to glucuronidate T4, so increased transcription of this enzyme could lead to decreased circulating T4 (Vansell and Klaassen, 2002). Another study indirectly supported this hypothesis by showing that administering 100 ppm Aroclor 1254, also for 7 days, increased the biliary excretion of T4G in rats (Vansell and Klaassen, 2001).

Induction of UDP-GT activity, increased transcription of UDP-GT mRNA, and increased biliary excretion of T4G often led to decreased circulating T4 concentrations. Circulating T4 concentrations were significantly reduced by administering 30 ppm Aroclor 1254, for as little as 7 days to rats (Liu et al., 1995) and by administering 30 ppm Aroclor 1254 for 21 days to mice (Hood et al., 2003). In both of these studies, 10 ppm was also used, but had no significant effects on circulating T4. Other studies on rats also showed that Aroclor 1254 reduced circulating T4 at concentrations of over 30 ppm (Barter and Klaassen, 1992b; Barter and Klaassen, 1994; Vansell and Klaassen, 2001).

Considering that Aroclor 1254 markedly decreases plasma T4 concentrations in rats, it is surprising that it (at 100-300 ppm in rats) usually has little or no effect on circulating TSH (Hood et al., 1999; Hood and Klaassen, 2000; Liu et al., 1995; Vansell and Klaassen, 2001). However, there are two exceptions. One study in rats, using 250 ppm Aroclor 1254, showed an increase in circulating TSH after 14 days (Barter and Klaassen, 1994). Another study showed a slight increase in TSH concentrations and a marked increase in thyroid follicular cell proliferation in mice after exposure to 100 ppm Aroclor 1254 for 21 days (Hood et al., 2003). Thyroid follicular cell proliferation indicates increased stimulation by TSH. Other effects of Aroclor 1254, which also are usually indicative of increased TSH production, were apparent even in experiments in which there was no significant increase in TSH production. These effects include increased thyroid gland weight (Barter and Klaassen, 1994), thyroid gland hypertrophy (Liu et al., 1995), and increased uptake of 131I by the thyroid gland (Barter and Klaassen, 1994; Liu et al., 1995). When negative feedback to the HPT axis occurs in response to decreased circulating T4 concentrations, one would expect to find increased TSH.
concentrations. However, these studies have mostly found no increase in TSH production; the reason for this is not understood.

In several studies using Aroclor 1254 in rats, measurements of circulating T3 were obtained. Three of these studies (200-300 ppm) revealed little or no change in T3 concentrations (Barter and Klaassen, 1994; Hood and Klaassen, 2000; Liu et al., 1995), and one (250 ppm) showed a significant decrease in circulating T3 (Barter and Klaassen, 1992b). Maintenance of circulating T3 concentrations may be due to increased activity of 5′-deiodinase (enzyme that converts T4 to T3). Also, glucuronidation of T4 is a major metabolic pathway compared to glucuronidation of T3, which may also explain why circulating T4 concentrations decrease while T3 concentrations are maintained (Liu et al., 1995).

Kanechlor-500. Kanechlor-500 is also a mixture of PCBs and has been found to decrease plasma THs. It was shown, 4 days after a single ip injection of 100 mg/kg, to significantly decrease circulating T4 in rats and mice (Kato et al., 2003). However, in this same study, UDP-GT (UGT1A1 and UGT1A6) activity toward T4 was induced in rats but not in mice. Although T4 concentrations were decreased, there were no significant effects on TSH concentrations. T3 concentrations were also examined, and mice, but not rats, showed significantly lower T3 concentrations after treatment with Kanechlor-500.

Dioxin-like PCB congeners. In addition to PCB mixtures, individual congeners have also been studied, although not as extensively as mixtures such as Aroclor 1254. PCB 126 (oral dose of 3 µg/kg/day for 4 days), one of the most toxic and the most dioxin-like congener, significantly decreased circulating T4 in rats, but up to 300 µg/kg/day for 4 days had no effect on mice. However, in both rats (at 10 µg/kg/day for 4 days) and mice (at 90 µg/kg/day for 4 days), T4 glucuronidation was increased. Hepatic UGT1A expression was significantly increased in response to PCB 126 in rats and only minimally in mice (Craft et al., 2002).

Non-dioxin-like PCB congeners. PCBs 110, 132, 149, and 153 are some of the congeners that have been studied for their effects on UDP-GT. These are all non-coplanar and therefore more likely less toxic than coplanar PCBs, but most environmental PCBs are non-coplanar congeners (Li et al., 2001). PCB 110 caused a significant decrease in circulating T4 in rats with an ip injection of 32 mg/kg and had no effect on UDP-GT activity (Li et al, 1998). A mixture of PCB 110 contaminated with PCB 126 caused a significant decrease in circulating T4 in rats with an ip injection of 32 mg/kg and induced UDP-GT activity with an ip injection of 4 mg/kg (Li et al, 1998). PCB 132 (ip injection of up to 96 mg/kg) in rats caused no significant changes in circulating T4, UDP- GT activity, TSH concentrations, or T3 concentrations (Li et al., 2001). PCB 149 (ip injection of 32 mg/kg) in rats significantly decreased circulating T4 (8 mg/kg caused no significant effects), but UDP-GT activity and TSH concentrations were not affected by up to 96 mg/kg PCB 149 (Li et al., 2001). PCB 153 significantly decreased circulating T4 in rats (oral dose of 9 mg/kg/day for 4 days) and mice (oral dose of 3 mg/kg for 4 days) and increased T4 glucuronidation in rats (oral dose of 90 mg/kg for 4 days) and mice (oral dose of 300 mg/kg for 4 days; Craft et al., 2002). In general, mammals appeared to be more sensitive to PCBs 110 and 153 than to PCBs 132 and 149.

Of the PCBs that have been studied in mammals, only Aroclor 1254, Kanechlor-500, PCB 126, and PCB 153 have both induced UDP-GT activity toward T4 and
decreased circulating $T_4$ concentrations. In general, however, mice appear to be slightly less sensitive to PCBs than rats.

**Birds**  
Compared to mammals, there have been relatively few studies on UDP-GT induction in birds. In a field study on common tern chicks (*Sterna hirundo*), there were no significant differences in UDP-GT activity or in $T_4$ concentrations in embryos in which the highest PCB concentration in yolksacs was 6.4 times higher than the lowest (Murk et al., 1994). In contrast, a laboratory study showed that Phenochlor, a PCB mixture, with an oral dose of 5 mg/bird/day for 40 days, increased UDP-GT activity in adult Japanese quail (*Coturnix japonica*; Riviere et al., 1978). In a study in our laboratory, chicken embryos (*Gallus domesticus*) were exposed *in ovo* to PCB 77 (0.64 ng/g egg) or PCB 126 (up to 0.80 ng/g egg), both dioxin-like PCBs. There were no significant changes in plasma TH concentrations or in UDP-GT activity, but the highest dose caused 40% mortality (McCleary, 2001). There is not enough information on UDP-GT induction by PCBs in birds to make generalizations. The contrasting results found in these studies may be due to the different PCBs, doses, routes of administration, and species and ages of animals.

Several studies have shown that even when circulating TH concentrations were not altered in birds exposed to environmental PCBs, enlarged thyroid glands and decreased thyroidal THs (thyroid gland TH content) were still evident. One of these studies showed that in herring gull (*Larus argentatus*) and Caspian tern chicks (*Sterna caspia*) in the Great Lakes across a wide range of PCB concentrations, in 1991 and 1992, plasma $T_4$ concentrations were not correlated to PCB concentrations (Grasman et al., 1996). A more recent study (in 2001), this time on adult herring gulls from high and low PCB concentrations in the Great Lakes, also showed no differences in plasma TH concentrations that correlated to PCB concentrations. However, this study did reveal enlarged thyroid glands and decreased TH stores in populations from more contaminated sites. From 1998 to 2000, compared with reference sites, herring gull pipping embryos and prefledglings from the Great Lakes had markedly decreased TH stores. In addition, a significant inverse relationship has been found between exposure to PCBs and thyroidal $T_4$. However, decreased TH stores did not always lead to organismal hypothyroidism (review, McNabb and Fox, 2003).

Several laboratory studies in birds have been conducted to determine the effects of PCBs on the thyroid gland and circulating TH concentrations. Some of these effects include decreased body temperatures, decreased circulating TH concentrations, enlarged thyroid glands, increased iodide uptake by the thyroid gland, and decreased femur lengths, all indicative of decreased thyroid function (review, McNabb and Fox, 2003). In one study, mourning doves (*Zenaida macroura carolinensis*) that were fed PCBs showed a trend of decreased metabolic rates and significant decreases in body temperature (Tori and Mayer, 1981). PCB 77 was also shown to decrease body temperature, as well as to cause goiter and decreased serum TH concentrations in ring doves (*Streptopelia risoria*) Spear and Moon, 1985). Aroclor 1254 has also been shown to cause goiter in guillemots (*Uria aalge*; Jefferies and Parslow, 1976). Another study showed that Alcolar (1260), a PCB, caused increased thyroid gland weight and increased iodide uptake by the thyroid gland in bobwhite quail (*Colinus virginianus*; Hurst et al., 1974). A mixture of Aroclors
1248, 1254, and 1260 fed to adult American kestrels (*Falco sparverius*) caused a significant decrease in plasma $T_3$ (Smits et al., 2002). Our lab has found trends supporting that PCBs may displace $T_4$ from transthyretin, a plasma TH binding protein (Maher and McNabb, unpublished).

Other laboratory studies on birds have shown contrasting results. PCBs have been shown to increase circulating TH concentrations in some studies and decrease them in other studies. In addition, femur lengths are sometimes longer and sometimes shorter compared to controls in response to PCB exposure. At high doses, Aroclors 1254 and 1242 have been shown to decrease plasma $T_4$ concentrations in chicken embryos (Gould et al., 1999), but Aroclor 1254 had no effect on $T_4$ in another study on chicken embryos (Gould et al., 1997). At low doses, Aroclor 1242 significantly increased plasma $T_4$ concentrations in chicken embryos (Gould et al., 1997), and an organochlorine mixture that included PCBs also increased plasma $T_4$ concentrations in adult ring doves (*Streptopelia risoria*; McArthur et al., 1983). Aroclors 1254 (Gould et al., 1997) and 1242 (at high doses only; Gould et al., 1997; Gould et al., 1999) had no effect on circulating $T_3$ concentrations, but they caused decreased femur lengths in chicken embryos. However, Gould et al. (1999) found that Aroclor 1254 had no effect on femur length in chicken embryos, and the same authors (1997) found that low doses of Aroclor 1242 actually increased femur lengths in chicken embryos. These discrepancies between studies are likely due to different types and doses of PCBs and differences in species and ages of animals, warranting further investigation on the effects of PCBs on avian UDP-GT activity and thyroid function.

In summary, the studies cited above show that PCBs have been studied fairly extensively for their effects on UDP-GT activity and thyroid function in mammals, but there have been very few studies done on avian UDP-GT induction by PCBs. Many PCBs, including Aroclor 1254, disrupt thyroid function in laboratory mammals, so it is assumed that they will have similar effects on birds. This is the focus of my research.
Chapter 2: Introduction

In wildlife such as fish-eating birds, polychlorinated biphenyls (PCBs) have been associated with developmental abnormalities that may be due to altered thyroid function (reviews, Fox, 1993; McNabb and Fox, 2003; Scanes and McNabb, 2003). This has stimulated research on how PCBs affect thyroid function, but most of the research has been done on laboratory mammals. In several such studies, PCBs caused organismal hypothyroidism as indicated by decreased circulating thyroxine ($T_4$), the predominant thyroid hormone (TH) produced by the thyroid (Barter and Klaassen, 1992b; Barter and Klaassen, 1994; Hood et al., 2003; Liu et al., 1995; Vansell and Klaassen, 2001). In mammals, PCBs increase the excretion of $T_4$ by two mechanisms. (1) PCBs induce uridine diphosphate-glucuronosyltransferase (UDP-GT), a hepatic phase II biotransformation enzyme that glucuronidates $T_4$ and thereby facilitates its excretion in bile (Barter and Klaassen, 1992b; Franklin and Yost, 2000). (2) PCBs displace $T_4$ from transthyretin, a TH binding protein in the blood, releasing free $T_4$, which may be metabolized and excreted (review, Brouwer et al., 1998). If these mechanisms increase excretion of $T_4$, circulating $T_4$ concentrations will decrease, potentially resulting in hypothyroidism.

Most of the studies on PCB effects on UDP-GT and thyroid function have used laboratory rodents. Aroclor 1254, an industrial mixture of PCBs, has been the most commonly used PCB in experiments testing the effects of PCBs. It has been shown to induce UDP-GT activity in both rats and mice (Barter and Klaassen, 1992b; Barter and Klaassen, 1994; Hood et al., 2003; Hood and Klaassen, 2000; Liu et al., 1995). In rats, Aroclor 1254 increases mRNA transcription of two isozymes of UDP-GT that are known to glucuronidate $T_4$ (Vansell and Klaassen, 2002), increases the biliary excretion of $T_4$ glucuronide ($T_4G$; Vansell and Klaassen, 2001), and decreases plasma $T_4$ (Barter and Klaassen, 1992b; Barter and Klaassen, 1994; Hood et al., 2003; Liu et al., 1995; Vansell and Klaassen, 2001).

Studies on PCB effects on avian UDP-GT induction are few in number compared to those on mammals. A laboratory study on adult Japanese quail (Coturnix japonica) showed that Phenochlor, a PCB mixture, increased UDP-GT activity (Riviere et al., 1978). Likewise, in another study on adult Japanese quail (Elliott et al., 1997), it was found that Aroclor 1254 induced activity of a phase I biotransformation enzyme (ethoxyresorufin O-deethylase; EROD). Phase II biotransformation enzymes found in microsomes (e.g., UDP-GT) often are induced in conjunction with the induction of phase I biotransformation enzymes (Parkinson, 2001). In contrast, in a laboratory study in which chicken embryos (Gallus domesticus) were exposed to PCBs 77 and 126, and in a field study on common tern chicks (Sterna hirundo) exposed to environmental PCBs, it was shown that there was no apparent relationship between PCB exposure and avian UDP-GT activity (McCleary, 2001; Murk et al., 1994).

In the only previous study showing PCB induction of avian UDP-GT activity, para-nitrophenol ($pNP$) was used as a substrate, so this assay was not specific to $T_4$. (For the UDP-GT assays used in mammalian studies, $T_4$ was used as substrate.) UDP-GT has many forms (at least 16 isozymes have been identified in rats; Vansell and Klaassen, 2002), and almost all of these isozymes glucuronidate $pNP$ (Franklin and Yost, 2000). It is unknown how many of these isozymes glucuronidate $T_4$, but in rats, at least two of
them act on $T_4$ (Vansell and Klaassen, 2002). Therefore, glucuronidation of $p$NP may not accurately reflect glucuronidation of $T_4$. For this reason, before studying the effects of PCBs on avian UDP-GT activity toward $T_4$, an assay that could measure UDP-GT activity toward $T_4$ in birds had to be developed.

PCB effects on avian thyroid function have differed in different studies. In one study, it was shown that Aroclor 1242 and Aroclor 1254 decreased circulating $T_4$ concentrations after 21 days in chicken embryos (Gould et al., 1999). In another study, Aroclor 1254 had no effect on circulating $T_4$ concentrations after 17 days and Aroclor 1242 increased circulating $T_4$ concentrations after 17 days in chicken embryos (Gould et al., 1997). Circulating $T_4$ concentrations were not altered in chicken embryos exposed to PCBs 77 and 126 or in common tern chicks exposed to environmental PCBs in the field (McCleary, 2001; Murk et al., 1994). Many of the differences in these results may be due to differences in the species used, the specific PCBs used, the dose administered, and the exposure time used. However, it is difficult without further investigation to determine which one of these factors or which combination of these factors is causing these differences in results.

The objectives for my study were (1) to develop an assay for measuring avian UDP-GT activity using labeled $T_4$ as substrate and (2) to determine the effects of Aroclor 1254 on UDP-GT induction and thyroid function in Japanese quail and to compare these effects to those in mice. In both species, if UDP-GT induction occurs, liver weight is expected to increase (due to hepatic enzyme induction) and plasma TH concentrations are expected to decrease due to increased excretion of $T_4$. In response to decreases in circulating THs, feedback activation of the hypothalamic-pituitary-thyroid (HPT) axis is expected to occur. Increased release of thyroid stimulating hormone (TSH) from the pituitary should then stimulate thyroidal TH production/release, thereby maintaining circulating TH concentrations at euthyroid levels. This process should continue until thyroidal TH stores are depleted, circulating THs are markedly decreased, and the birds are overtly hypothyroid. If TSH increases are sustained, they will stimulate growth of the thyroid gland.
Chapter 3: Materials and Methods

Experimental Design

*Development of an assay for measuring avian UDP-GT activity toward T₄.*

Development of this assay included validating it by defining reaction conditions which were not limiting (i.e., in which enzyme activity was linear with enzyme concentration and incubation time). I altered three factors (substrate concentration, incubation time, and enzyme concentration) to maximize enzyme activity.

*Experiment 1: UDP-GT induction in Japanese quail and mice after 5-day exposure to Aroclor 1254.* Twelve-day-old Japanese quail (*Coturnix japonica*) and Balb/c weanling mice were dosed by oral gavage once with vehicle (corn oil), 250 mg Aroclor 1254/kg body weight, or 500 mg Aroclor 1254/kg body weight. All animals were sacrificed five days after dosing. In the control groups, there were 5 mice and 10 quail. Five mice and 5 quail received 250 mg/kg Aroclor 1254, and 5 mice and 4 quail received 500 mg/kg Aroclor 1254.

*Experiment 2: UDP-GT induction in Japanese quail after 5-day or 21-day exposures to Aroclor 1254.* Twelve-day-old Japanese quail were dosed by oral gavage once with vehicle (safflower oil) or 500 mg Aroclor 1254/kg body weight. Twenty-six animals (13 control; 13 treatment) were sacrificed five days after dosing, and 23 (12 control; 11 treatment) were sacrificed 21 days after dosing.

Development of an Assay for Measuring Avian UDP-GT Activity Toward T₄

McCleary (2001) began development of this assay by modifying/combining the methods of Beetstra et al. (1991), and Barter and Klaassen (1992a), which describe an assay previously validated for use on rodents. I further modified McCleary’s methods for use in quail.

*Liver homogenate (HOM) preparation.* Frozen livers from adult Japanese quail were thawed and homogenized at a ratio of 1.0 g liver to 2.0 ml buffer (50 mM Tris-HCl w/ 150 mM KCl; pH 7.4) in a Radnoti glass homogenizer by 10 strokes of the glass pestle. The HOM was filtered through one layer of nylon organdy and stored at -80°C. This is referred to as full strength HOM and was diluted later for use in the assay. Tris-HCl and KCl were supplied by Fisher Scientific (Pittsburgh, PA). The homogenizer was from Radnoti Glass Technology, Inc. (Monrovia, CA).

*UDP-GT assay.* Each sample was prepared in triplicate. The following components were combined in 1.5 ml microcentrifuge tubes: 50 µl HOM (dilutions between 1:0.67 and 1:32; full strength HOM:buffer, v/v), 80 µl buffer (100 mM Tris HCl, 13.33 mM MgCl₂, 0.033% Brij 58, and 1.87 mM saccharic acid-1,4-lactone; pH 7.4), 20 µl T₄ in H₂O (0.1-400 µM), 20 µl ¹²⁵I-T₄ (~100,000 cpm) in buffer (50 mM Tris-HCl w/ 150 mM KCl; pH 7.4), and 30 µl 33.33 mM uridine diphospho-glucuronic acid (UDPGA). Reaction mixtures were vortexed and incubated between 10 and 120 min in a 37°C water bath. Immediately afterwards, the reaction mixtures were placed in an ice-water bath and 200 µl ice-cold methanol was added to each tube to terminate the reaction.
HOM dilution, T4 concentration, and incubation time were all determined during validation of the assay.

Reaction mixtures were centrifuged at 2000g for 20 min at 0°C. The supernatant (375 µl) was removed, and the pellet was resuspended with 500 µl ice cold methanol and recentrifuged at 10,000g for 20 min at 0°C. This supernatant (530 µl) was added to the first supernatant collected, 200 µl HCl was added, and the mixture was vortexed.

A 1000 µl aliquot of this extract mixture was loaded onto Sephadex LH-20 columns (2.0 ml bed volume; Poly-Prep columns) that had been prewashed with 1 x 2.0 ml 0.1 N HCl, 2 x 2.0 ml 0.1 N NaOH/100% ethanol (1:1, v/v), and 2 x 2.0 ml 0.1 N HCl. The prewashes ensured that the columns were free of the phosphate buffer in which they were stored and ready for the eluants used during separation of the components of the mixture. First, free iodide was eluted with 7 x 1.5 ml 0.1 N HCl, then T4G was eluted with 10 x 1.5 ml 0.1 N double distilled H2O, and last, T4 was eluted with 8 x 1.5 ml 0.1 N NaOH/100% ethanol (1:1, v/v). The radioactivity in each fraction was counted for 4 min (Fig. 5).

Tris-HCl, KCl, methanol, HCl, and NaOH were supplied by Fisher Scientific (Pittsburgh, PA). T4, UDPGA, MgCl2, Brij 58, saccharic acid-1,4-lactone, and Sephadex LH-20 resin were obtained from Sigma-Aldrich (St. Louis, MO). Radiolabeled T4 (1250 µCi/µg 125I-T4) was supplied by PerkinElmer (Wellesley, MA), ethanol was supplied by AAPER Alcohol and Chemical Co. (Shelbyville, KY), and the Poly-Prep chromatography columns were supplied by Bio-Rad Laboratories (Hercules, CA).

Enzyme activity was determined by calculating the proportion of T4 that was glucuronidated (ratio of radioactivity in the water fractions to the sum of the radioactivity in the water and alkaline ethanol fractions). This proportion and the fact that there were 30,000 pmol cold T4 and 1.85 mg liver in each reaction mixture made it possible to calculate pmol T4G/mg liver per min. Blanks were subtracted from each result.

Aroclor 1254

Aroclor 1254 was supplied by Accustandard (New Haven, CT) for Experiment 1 and was kindly donated by Robert Letcher of Environment Canada (Ottawa, ON) for Experiment 2. Hexane was donated by Jean Cobb in the Department of Biochemistry at Virginia Tech, and nitrogen gas (Airgas; Radford, VA) was donated by Bobbie Niederlehner (Virginia Tech Department of Biological Sciences).

For Experiment 1, Aroclor 1254 was dissolved in corn oil. For Experiment 2, Aroclor 1254 was dissolved in hexane, mixed with safflower oil, and the hexane was evaporated with nitrogen gas. For controls, the same procedure was used, except without the Aroclor 1254.

Animals

Animal care and maintenance. Twelve-day-old Japanese quail (Coturnix japonica) were banded with individually numbered plastic legbands. Each treatment group was housed in a wire mesh game bird brooder cage (35.5” x 28” x 9”) on a 16-hour
light, 8-hour dark photoperiod. Brooder temperature adjacent to the heat lamp was 37-38°C the first week after hatching and was gradually decreased to 27-29°C by the fifth week. Quail were provided with water and chick starter food ration (20% protein; Big Spring Mill, Inc.; Elliston, VA) ad libitum.

Mice in Experiment 1 were marked uniquely on their tails with a permanent marker. Each treatment group was housed in a plastic Econo-Cage (11” x 6.75” x 5”) with a wire-mesh Econo-Cage lid. Pine shavings were used as bedding. Animals were maintained at 18-24°C on a 12-hour light, 12-hour dark photoperiod. Mice were provided with water and Harlan Tecklad Rodent Diet (18% protein; Madison, WI) ad libitum.

All animals were cared for in accordance with the policies of the Animal Care Committee of Virginia Tech.

Dosing animals with Aroclor 1254. Quail were weighed and then dosed by oral gavage using a 1 ml syringe with rubber tubing (4 mm diameter; ~ 1” length). Mice were weighed and then dosed by oral gavage using a 1 ml syringe with a PS 22 gauge gavage needle (donated by David Gemmell of the Virginia Tech Laboratory Animal Resources). Aroclor 1254 in corn oil or safflower oil was vortexed before each dose. All animals were dosed with 200 µl vehicle/25 g body weight, but for Experiment 2, doses were determined by mass rather than by volume.

Sampling. Quail were sacrificed by decapitation, and trunk blood was immediately collected in heparinized micro-hematocrit capillary tubes. Mice were sacrificed by cervical dislocation. Immediately afterwards, the chest was opened and blood was collected directly from the heart using heparinized micro-hematocrit capillary tubes. Plasma was separated and stored at –20°C. Each animal was weighed, and the thyroid glands and livers were removed, weighed, and stored at –80°C.

PCB-contaminated waste disposal. All bedding, waste, and carcasses contaminated with Aroclor 1254 were disposed of in accordance with university chemical safety procedures.

Analytical Techniques

Thyroidal THs. Thyroidal THs (thyroid gland TH content) was measured by the method of McNabb and Cheng (1985). This involved incubating each thyroid gland in 350 µl of a digestion mixture (76.78 mM Tris-HCl, 5.0 mM glutathione, 2.5 mM 6-n-propyl-2-thiouracil, and 1% Triton-X-100) containing 20 mg Pronase for 24 hours in a 37°C water bath. To terminate the reaction, 1.0 ml 100% ethanol was added to each digestion tube and the tubes were vortexed. To extract THs, the tubes were placed at -20°C for 24 hours and then centrifuged for 8 min at 13,500 g at -4°C. Supernatant was collected and stored at -20°C for later analysis by radioimmunoassay (RIA) as described below. Tris-HCl was supplied by Fisher Scientific (Pittsburgh, PA). Glutathione, 6-n-propyl-2-thiouracil, and Triton-X-100 were supplied by Sigma-Aldrich (St. Louis, MO). Pronase bacterial protease from Streptomyces griseus was supplied by CalBiochem (San Diego, CA).

Radioimmunoassay (RIA). Thyroxine (T4) and triiodothyronine (T3) concentrations in plasma and thyroid gland extracts were measured using a double
antibody RIA as described by McNabb and Hughes (1983). Standards were prepared in hormone-free chicken plasma for plasma analyses or in 75% ethanol for thyroid gland extract analyses. Assay volumes were 25 µl for T₃ assays and for the ethanol-based T₄ assay; 12.5 µl was used for the plasma T₄ assay. Precision for RIAs was 10.6% ± 4.7 (± 2 SE as a % of the mean) for 10 replicates. Standardized control sera were run with each set of samples to evaluate the performance of the assay. The RIA was validated for use on quail and mouse plasma and thyroid extracts.

T₃ primary antibody was purchased from Sigma-Aldrich (St. Louis, MO), T₄ primary antibody was purchased from Fitzgerald Industries International, Inc. (Concord, MA), ¹²⁵I-T₄ and ¹²⁵I-T₃ (1250 µCi/µg) were supplied by PerkinElmer (Wellesley, MA), and carrier immunoglobulin was supplied by Antibodies Inc. (Davis, CA). T₄ and T₃ immunoassay control sera were supplied by Randox Laboratories (Crumlin, Co. Antrim, UK). Dr. John McMurtry (USDA; Beltsville, MD) kindly supplied secondary antibody.

Statistics

Analysis of variance (ANOVA) was used to analyze all data, followed by multiple comparisons of the means with Tukey-Kramer adjustments of the p-values. For Experiment 1, factors were dose and species. For Experiment 2, factors were dose, exposure time, and sex. All statistics were performed using SAS (Cary, NC).
Chapter 4: Results

Development of an Assay for Measuring Avian UDP-GT Activity Toward T₄

To validate the assay, substrate (T₄) concentration, liver homogenate (HOM) dilution, and incubation time were altered until enzyme activity was linear with both HOM dilution and incubation time at abundant substrate concentrations. In a study to develop a UDP-GT assay for mammalian tissues, it was found that there was no increase in enzyme activity when concentrations over 100 µM T₄ were used in the assay, so they used 150 µM T₄ (Barter and Klaassen, 1992a). This same concentration was used in a UDP-GT assay for a study on mice in the same laboratory (Hood et al., 2003). In the present study, several T₄ concentrations (0.1-400 µM T₄, final concentrations in reaction mixture) including trace amounts of ¹²⁵I- T₄ were compared. It was confirmed that 150 µM T₄ yielded the maximum enzyme activity. Tests of HOM dilutions indicated that a 1:8 (v/v) dilution of full strength HOM yielded the least variable enzyme activity under conditions where enzyme activity was linear with time. Tests of incubation times indicated that enzyme activity was linear with time and had low variability from 20-40 min. The final optimal assay conditions used for all sample analyses for the Aroclor 1254 experiments included 150 µM T₄, a 1:8 dilution of HOM, and a 30 min incubation time.

Effects of Aroclor 1254 on Mice

Body weight. Control and treatment groups of female mice in Experiment 1 did not differ in body weight or weight gain for either Aroclor 1254 dose. Body weight of controls ranged from 10.9-16.5 g after five days. (All statistical data are shown in Tables 1 and 2.)

UDP-GT activity and liver weight. UDP-GT activity per unit weight of liver was increased significantly in mice at both doses of Aroclor 1254 after five days compared to controls (250 mg/kg dose: 241% of controls; 500 mg/kg: 478% of controls; Fig. 6a). Liver weight as a percent of body weight also was increased significantly at both doses of Aroclor 1254 after five days compared to controls (250 mg/kg: 123% of controls; 500 mg/kg: 213% of controls; Fig. 6b). Liver weights of controls ranged from 0.5-0.9 g. The increase in both UDP-GT activity per unit weight of liver and in liver weight as a percent of body weight led to an increase in UDP-GT activity per liver (250 mg/kg: 294% of controls; 500 mg/kg: 963% of controls; data not shown).

Thyroid function. Plasma T₄ concentrations were significantly decreased after five days of exposure to 250 mg/kg (50% of controls) and 500 mg/kg (37% of controls) Aroclor 1254 compared to controls (Fig. 6c). Plasma T₃ concentrations were not altered at the lower dose, but they were significantly increased at the higher dose (166% of controls) after five days of exposure compared to controls (Fig. 6d). The ratio of plasma T₃:T₄ tended to increase with five days of exposure to Aroclor 1254 (controls: 1:55; 250 mg/kg: 1:23; 500 mg/kg: 1:12).

Thyroid gland weight as a percent of body weight and thyroidal T₄ were not significantly altered in mice at either dose after five days, although thyroid gland weight
tended to increase (data not shown; control thyroid gland weight ranged from 1.5-2.3 mg; control thyroidal T_4 ranged from 67-164 ng/thyroid pair). Thyroidal T_3 after five days of exposure was significantly increased at 250 mg/kg (179% of controls) and at 500 mg/kg (224% of controls) Aroclor 1254 compared to controls (data not shown; controls ranged from 41-57 ng/thyroid pair). At the lower dose, T_3 per unit weight of thyroid pair tended to increase, and at the higher dose, there was a significant increase in T_3 per unit weight of thyroid pair after five days of exposure compared to controls (data not shown). The thyroidal ratio of T_3:T_4 increased slightly with five days of exposure to Aroclor 1254 (controls: 1:2; 250 mg/kg: 1:1; 500 mg/kg: 1:1).

Effects of Aroclor 1254 on Quail

Sex and body weight. The sample sizes of quail were small in Experiment 1, so the effect of sex could not be analyzed. There were no significant differences between sexes for any of the variables measured in quail in Experiment 2. Control and treatment groups did not differ in body weight or weight gain in either quail experiment. Body weight of controls ranged from 18-56 g after five days and 39-72 g after 21 days.

UDP-GT activity and liver weight. UDP-GT activity per unit weight of liver was increased significantly in quail after five and 21 days at 500 mg/kg Aroclor 1254 compared to controls (5 days, Experiment 1: 300% of controls, data not shown; 5 days, Experiment 2: 156% of controls; 21 days: 164% of controls; Fig. 7a). At 250 mg/kg Aroclor 1254 after five days, there was a trend to increased UDP-GT activity per unit weight of liver (217% of controls, data not shown). Liver weight as a percent of body weight was not altered at 250 mg/kg after 5 days but was significantly increased at 500 mg/kg Aroclor 1254 after 5 days in both experiments (Experiment 1: 142% of controls, data not shown; Experiment 2: 140% of controls; Fig. 7b). Liver weight as a percent of body weight also was not increased at 500 mg/kg after 21 days (Fig. 7b). Liver weights of controls ranged from 0.7-1.8 g after five days and 1.1-2.8 g after 21 days. UDP-GT activity per liver was increased significantly at all doses and exposure times compared to controls (5 days, 250 mg/kg: 249% of controls; 5 days, 500 mg/kg, Experiment 1: 381% of controls; 5 days, 500 mg/kg, Experiment 2: 200% of controls; 21 days, 500 mg/kg: 166% of controls; data not shown).

Thyroid function. Plasma T_4 concentrations were not altered significantly but tended to decrease with dose of Aroclor 1254 at both doses and exposure times compared to controls (Fig. 7c). Plasma T_3 concentrations were not altered at 250 mg/kg after five days but were significantly decreased at 500 mg/kg Aroclor 1254 after five days compared to controls in both experiments (Experiment 1: 56% of controls, data not shown; Experiment 2: 64% of controls; Fig. 7d). Plasma T_3 concentrations were not altered at 500 mg/kg after 21 days compared to controls (Fig. 7d). The ratio of plasma T_3:T_4 did not change significantly at any dose or exposure time compared to controls (mean of 1:3).

Thyroid gland weights as a percent of body weight were increased significantly in quail at 250 mg/kg and 500 mg/kg Aroclor 1254 after five days compared to controls in Experiment 1 but were not altered at 500 mg/kg Aroclor 1254 after five or 21 days in Experiment 2 (data not shown; controls ranged from 1.8-6.8 mg after five days and 1.9-
8.4 mg after 21 days). Thyroidal T₄ and T₃ were not significantly altered at either Aroclor 1254 dose or exposure time compared to controls (Fig. 8). However, thyroidal T₃ tended to decrease at the higher dose after 21 days. The thyroidal ratio of T₃:T₄ did not change at any dose or exposure time compared to controls (mean of 1:129).

**Comparison of Quail and Mice**

*UDP-GT activity and liver weight.* In control groups, quail UDP-GT activity per unit weight of liver was 22% of that of mice (p = 0.0003). UDP-GT activity per liver was not significantly different between species, although it tended to be higher in mice than quail. In controls, liver weight as a percent of body weight was significantly larger in mice than in quail (p = 0.0008).

Enzyme activity in response to Aroclor 1254 was less in quail than in mice. At 250 mg/kg Aroclor 1254, quail UDP-GT activity per unit weight of liver increased (but not significantly) to 217% of controls, and mouse UDP-GT activity per unit weight of liver increased significantly to 241% of controls. At 500 mg/kg, quail UDP-GT activity per unit weight of liver increased significantly to 300% of controls, and mouse enzyme activity increased significantly to 478% of controls. Liver weight as a percent of body weight was significantly increased only in mice (123% of controls) at 250 mg/kg Aroclor 1254, but it was significantly increased in both species at 500 mg/kg Aroclor 1254 after five days (quail: 142% of controls; mice: 213% of controls).

*Thyroid function.* Organismal thyroid status, as indicated by plasma T₄ concentrations, was not altered in quail, but it was significantly decreased in mice at both doses of Aroclor 1254 after five days. Plasma T₃ concentrations were significantly decreased in quail at 500 mg/kg Aroclor 1254 after five days of exposure, but plasma T₃ concentrations were significantly increased in mice at the same dose and exposure time. In controls, the ratio of plasma T₃:T₄ for quail was 1:3, but in mice, it was 1:55. Thyroid gland weight as a percent of body weight was increased significantly in quail but not in mice at either dose of Aroclor 1254. Thyroidal T₄ was not altered for either species at either dose. Thyroidal T₃ was not altered in quail but was significantly increased in mice at both doses; thyroidal T₃ per unit weight of thyroid pair was increased only in mice and only at 500 mg/kg Aroclor 1254. In controls, thyroidal ratio of T₃:T₄ for quail was 1:142, whereas it was 1:2 for mice.
Chapter 5: Discussion

As I hypothesized, PCB exposure induced UDP-GT activity toward T_4 and increased liver weight in birds. However, although this enzyme induction would have been expected to increase T_4 excretion and cause hypothyroidism, thyroid function was only slightly altered (i.e., the birds remained euthyroid). Decreased thyroid function in mice exposed to PCBs was equivalent to the responses of rodents in similar studies.

Because the glucuronidation of T_4 facilitates T_4 excretion in bile, it is generally assumed that UDP-GT induction by PCBs should result in hypothyroidism (review, McNabb and Fox, 2003). However, there have been no studies in which the links between PCB exposure, UDP-GT induction and thyroid function have been addressed in birds using a validated, T_4-specific UDP-GT assay. Increased avian UDP-GT activity and increased liver weight have been shown in response to PCBs in one other study (Riviere et al., 1978). However, the assay used in that study used a substrate, ρNP, that is glucuronidated by almost all UDP-GT isozymes. As there have been no previous studies of individual UDP-GT isozymes or of T_4 glucuronidation in birds, it remained unclear whether T_4 glucuronidation was enhanced by PCB exposure in birds. My study demonstrates that PCBs induce UDP-GT isozymes in birds that are specific to T_4, as measured by the assay that I developed and validated.

In quail, total liver UDP-GT capacity increased significantly in all treatment groups compared to controls. This increased capacity is the result of the combination of increased enzyme activity per unit weight of liver and increased liver weight (as a percent of body weight). UDP-GT activity per unit weight of liver increased in all treatment groups but was only significant at the higher dose after either exposure period. After five days of exposure to the lower dose, the trend to increased UDP-GT activity per unit weight of liver combined with the trend to increased liver weight (as a percent of body weight) resulted in significantly increased UDP-GT capacity for the whole animal. Although liver hypertrophy did not occur after 21 days, both UDP-GT activity per unit weight of liver and UDP-GT activity per liver (capacity for whole animal) increased significantly. It is possible that by 21 days after dosing, enough of the dose may have been metabolized and excreted that the effects of PCBs were reduced compared to five days after dosing. Other studies have shown that PCBs cause liver hypertrophy in birds (Elliott et al., 1997; Fowles et al., 1997; Riviere et al., 1978), which is consistent with my results after five days of exposure to the higher dose.

It has generally been assumed in rodents that circulating THs decrease in response to increased UDP-GT activity toward T_4. T_4G is more likely than T_4 to be excreted in the bile and consequently to be lost from the body, which should decrease circulating THs. However, my results in quail show that, despite the 1.7-3.8x increase in UDP-GT activity per liver (both exposure times and both doses), circulating T_4 only tended to decrease (not significant) and circulating T_3 decreased significantly. In rats, T_4G in the bile has been shown to increase in response to Aroclor 1254 (Bastomsky, 1974), but T_4G is not necessarily fully excreted after reaching the bile because deconjugation and reabsorption of the T_4 in the gut is possible (review, DiStefano, 1988). We did not measure biliary or fecal excretion of T_4, and therefore, it is a possibility that not all of the T_4G produced was excreted.
The decreased plasma $T_3$ concentrations in quail in both experiments at the higher dose after five days of exposure may be due to decreased activity of hepatic outer-ring deiodinase type-I activity (ORD), as occurred in response to Aroclor 1254 exposure in chicken embryos (Gould et al., 1999). However, in Gould’s study, circulating $T_4$ concentrations were decreased but circulating $T_3$ concentrations were maintained. The differences in circulating TH concentrations between the studies cited above and the present study may be due to differences in developmental age of the birds.

Because circulating $T_4$ concentrations tended to decrease and circulating $T_3$ concentrations decreased significantly, I expected activation of the HPT axis to occur with feedback leading to increased TSH release from the pituitary. Increased release of TSH should stimulate thyroid gland hormone production and release and thyroid gland growth. If HPT axis activation continues for long enough, stored $T_4$ released in response to TSH should eventually deplete thyroid gland $T_4$ stores. In Experiment 2, thyroid gland hypertrophy did not occur and thyroidal $T_4$ was not decreased, possibly because HPT axis activation was not marked enough to have an effect on thyroid gland hypertrophy or release of stored $T_4$. However, HPT axis activation (thyroid gland hypertrophy) did occur in quail in Experiment 1, even though thyroidal $T_4$ was still not altered. The reason for this difference between experiments is unclear. Similar to Experiment 2 quail, in the present study on mice and in past studies on rodents (Barter and Klaassen, 1994; Hood et al., 2003; Hood et al., 1999; Hood and Klaassen, 2000; Liu et al., 1995; Vansell and Klaassen, 2001), it appears that the HPT axis was not activated in response to decreased circulating $T_4$. There are two hypotheses that may explain the absence of HPT axis activation, which are discussed below in relation to both quail and mice.

In mice exposed to Aroclor 1254, as I hypothesized, UDP-GT activity and liver weight were increased significantly and there were resultant significant decreases in plasma $T_4$ concentrations five days after dosing. These results are congruent with other studies of laboratory rodents exposed to Aroclor 1254 that have shown increased UDP-GT activity and liver weight (Barter and Klaassen, 1992b; Barter and Klaassen, 1994; Hood and Klaassen, 2000; Liu et al., 1995) and decreased circulating $T_4$ concentrations (Barter and Klaassen, 1992b; Barter and Klaassen, 1994; Hood et al., 2003; Liu et al., 1995; Vansell and Klaassen, 2001). Because circulating $T_4$ concentrations were decreased in mice in the present study, HPT axis activation (i.e., stimulation of TSH release from the pituitary) and consequent thyroid gland hypertrophy should have occurred. Thyroid gland size was not altered by PCB exposure in the present study, and thus, it appears that HPT axis activation did not occur. The lack of depletion of thyroidal $T_4$ compared to controls in my study also indicates that the HPT axis was not activated. Decreased circulating $T_4$ concentrations should have resulted in increased TSH due to negative feedback, which in turn should have caused the release of stored thyroidal THs. These unexpected results are consistent with similar studies in rats or mice that have shown that although UDP-GT activity was induced and plasma $T_4$ concentrations were decreased by Aroclor 1254, TSH was either not altered significantly (Hood et al., 1999; Hood and Klaassen, 2000; Vansell and Klaassen, 2001) or was slightly increased (Barter and Klaassen, 1994; Hood et al., 2003; Liu et al., 1995).

There are two hypotheses to explain the apparent absence of HPT axis activation in both quail and mice. (1) PCBs may disrupt the HPT axis by inhibiting the pituitary or the hypothalamus, thus preventing a feedback response circulating THs, as has been
suggested by Hood and Klaassen (2000) and Liu et al. (1995). (2) PCBs may directly inhibit the thyroid gland. It has been shown in rats that the thyroidal response to exogenous TSH was reduced in response to PCBs (Byrne et al., 1987). This suggests that PCBs may inhibit the thyroid gland directly rather than inhibiting function of the HPT axis. Other studies on rats have shown that PCBs have been associated with alterations of thyroid gland histology, and it has been speculated that these alterations may reflect inhibition of the release of THs (Collins and Capen, 1980; Collins et al., 1977). How PCBs may inhibit the HPT axis or the thyroid gland itself remains unclear.

The increase in thyroidal T3 in PCB-exposed mice in my study was surprising because decreased circulating T4 concentrations should have activated the HPT axis (TSH release), which should have stimulated TH release from the thyroid gland. Because it appears that the HPT axis was not activated, I would expect thyroidal T3 to remain unaltered. Therefore, it is unclear why thyroidal T3 was increased.

I did not expect thyroidal or circulating T3 concentrations to increase in PCB-exposed mice in my study. Circulating T3 concentrations have been shown to decrease in other studies (but not proportionately to decreases in circulating T4 concentrations) with exposure to Aroclor 1254 in rats or mice (Barter and Klaassen, 1992b; Barter and Klaassen, 1994; Hood et al., 2003; Hood et al., 1999; Liu et al., 1995; Vansell and Klaassen, 2001). Only one study showed that Aroclor 1254 had no effect on circulating T3 concentrations (Hood and Klaassen, 2000). All but one of these studies involved rats, and the other involved male mice (Hood et al., 2003). In the present study, female mice were used, and they were slightly younger and of a different strain from the male mice used in the other study. These differences in species, sex, strain, and age may account for differences in circulating T3 concentrations between past studies and the present study. The overall response of mice to Aroclor 1254, including UDP-GT induction, was similar to that of other studies in which rodents were exposed to Aroclor 1254 (Barter and Klaassen, 1992b; Barter and Klaassen, 1994; Hood et al., 2003; Hood and Klaassen, 2000; Liu et al., 1995).

In conclusion, increased total liver UDP-GT capacity occurred in all PCB treatment groups of quail, and an assay has been validated for measuring UDP-GT activity toward T4 in quail. Because quail had lower UDP-GT induction than mice, it is expected that any decreases in thyroid function would be less in quail than those in mice. Although quail showed some alteration in thyroid function in response to UDP-GT induction, they remained euthyroid. In contrast, mice became hypothyroid in response to UDP-GT induction by PCBs. It is unclear how the HPT axis is affected (little or no effect) in quail in response to PCBs, and HPT axis activation appears to be inhibited in mice. Studies using higher doses and longer exposure times to Aroclor 1254 may further clarify some of the differences between birds and mammals in the effects of PCBs on thyroid function.
Literature Cited


Table 1. Statistical data for comparisons between control and treatment groups of Japanese quail and Balb/c mice after a 5-day exposure to Aroclor 1254 in Experiment 1.

<table>
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<th>Aroclor 1254 dose (mg/kg)</th>
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<td>UDP-GT activity</td>
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<td>65.52 2.7 ± 0.2</td>
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<td>(pmol/mg liver*min)</td>
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<td>6.5 ± 0.2 &lt;0.0001</td>
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<td>12.9 ± 1.8 0.0109</td>
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<td>Liver weight as % body</td>
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<td>UDP-GT activity</td>
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<td>13.31 ± 1.02 0.0126</td>
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<td>60.64 93.9 ± 3.3</td>
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<td>0.6147 0.0388</td>
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<td>Thyroidal T4 (ng/animal)</td>
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<td>0.95 62 ± 14</td>
<td>50 ± 10</td>
<td>70 ± 8</td>
<td>0.8038</td>
<td>66 ± 14</td>
<td>0.9673</td>
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<td>Thyroidal T3 (ng/animal)</td>
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<td>82.5 ± 5.1</td>
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<td>14.54 46.0 ± 5.1</td>
<td>82.5 ± 5.1</td>
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<td>Thyroidal T3 (ng/mg thyroid)</td>
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<td>45.2 ± 3.2</td>
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<td>9.02 26.3 ± 4.0</td>
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§P-values indicate comparisons between control and treatment groups.
Table 2. Statistical data for comparisons between control and treatment groups of Japanese quail in Experiment 2.

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<th>Aroclor 1254 dose (mg/kg)</th>
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<th>21 day exposure</th>
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<td>0</td>
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<td>Body weight increase (g)</td>
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<td>UAV-GT activity (pmol/mg liver*min)</td>
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<td>1.6 ± 0.1</td>
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<td>Liver weight as % body weight</td>
<td>32.96</td>
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<tr>
<td>UAV-GT activity (pmol/liver*min)</td>
<td>32.59</td>
<td>1564 ± 133</td>
<td>3130 ± 347</td>
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<tr>
<td>Plasma T&lt;sub&gt;4&lt;/sub&gt; (ng/ml)</td>
<td>0.21</td>
<td>8.7 ± 1.2</td>
<td>6.5 ± 0.9</td>
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<tr>
<td>Plasma T&lt;sub&gt;3&lt;/sub&gt; (ng/ml)</td>
<td>3.40</td>
<td>3.46 ± 0.26</td>
<td>2.21 ± 0.39</td>
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<td>Thyroid gland weight as % body weight</td>
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<td>Thyroidal T&lt;sub&gt;4&lt;/sub&gt; (ng/animal)</td>
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<td>1184 ± 208</td>
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<td>Thyroidal T&lt;sub&gt;4&lt;/sub&gt; (ng/mg thyroid)</td>
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<td>418 ± 44</td>
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<td>Thyroidal T&lt;sub&gt;3&lt;/sub&gt; (ng/mg thyroid)</td>
<td>0.14</td>
<td>1.31 ± 0.18</td>
<td>1.22 ± 0.15</td>
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*P-values indicate comparisons between control and treatment groups.
Figure 1. Basic structure of a PCB, with numbers indicating where chlorines may be substituted.

Figure 2. Structure of thyroxine (T₄).

Figure 3. Structure of triiodothyronine (T₃).
Figure 4. Hypothalamic-pituitary-thyroid (HPT) axis.
Figure 5. Profile of radioactivity recovery during column chromatography. a) All washes are shown. Washes 1-7: HCl (I); washes 8-17: H$_2$O (T$_4$G); washes 18-25: NaOH/100% ethanol (1:1, v/v; T$_4$). b) Only H$_2$O washes (8-17) are shown on a smaller scale.
Figure 6. Effects of Aroclor 1254 on Balb/c mice (Experiment 1): a) UDP-GT activity (pmol T₄G per mg liver per min); b) liver weight as a percent of body weight; c) plasma T₄ concentrations; d) plasma T₃ concentrations. Each value represents the mean ± SE of 5 mice. Asterisks indicate significant differences from controls (*p < 0.05; **p < 0.01).
Figure 7. Effects of Aroclor 1254 on Japanese quail (Experiment 2): a) UDP-GT activity (pmol T\textsubscript{4}G per mg liver per min); b) liver weight as a percent of body weight; c) plasma T\textsubscript{4} concentrations; d) plasma T\textsubscript{3} concentrations. Each value represents the mean ± SE of 11-13 quail. Asterisks indicate significant differences from controls (*p < 0.05; **p < 0.01).
Figure 8. Effects of Aroclor 1254 on Japanese quail thyroid gland hormone content (Experiment 2). Control values are different due to differences in ages of the birds. Each value represents the mean ± SE of 11-13 quail.
Curriculum Vitae

Catherine Marie Webb

Education
M.S., Biological Sciences, July, 2006. Virginia Tech, Blacksburg, Virginia
  Current Grade Point Average: 3.78/4.0
  Master’s Thesis: *Effects of Aroclor 1254 on UDP-GT Activity and Thyroid Function in Japanese Quail*
  Graduated Magna Cum Laude
  Grade Point Average: 3.75/4.0
  Major Grade Point Average: 3.69/4.0
  Senior Thesis: *The Effects of Malathion on Growth and Development of Xenopus laevis Tadpoles*

Employment
*Graduate Teaching Assistant*, Virginia Tech Department of Biological Sciences, Blacksburg, VA (August, 2003-May, 2006)
*Freshman Biology Laboratory Technician*, Virginia Tech Department of Biological Sciences, Blacksburg, VA (May, 2005-August, 2005)
*Peer Tutor*, Maryville College Learning Center, Maryville, TN (August, 2000-May, 2003)
*Pet Care Technician*, Concord Veterinary Hospital, Knoxville, TN (June, 2000-August, 2002)

Honors and Awards
Society of Environmental Toxicology and Chemistry North America 26th Annual Meeting; 3rd place in Student Poster Presentation (Fall, 2005)
A. Randolph Shields Award (outstanding senior in biology at Maryville College) (Spring, 2003)
Omicron Delta Kappa National Leadership Honor Society (Fall, 2001-Spring, 2003)
Maryville College Dean’s List (Fall, 1999-Spring, 2003)
Maryville College Dean’s Scholarship (Fall, 1999-Spring, 2003)
National Dean’s List (1999-2000)
All-American Scholar Collegiate Award (1999-2000)
Grants
Women and Minority Artists and Scholars Lecture Series (with Anne McNabb); Sep., 2005; $500
Society of Environmental Toxicology and Chemistry Student Travel Award; Sep., 2005; $360
Graduate Research Development Project, Virginia Tech; May, 2005; $150
Virginia Tech Department of Biology matching grant; May, 2005; $150
Graduate Research Development Project, Virginia Tech; May, 2004; $225
Virginia Tech Department of Biology matching grant; May, 2004; $225

Refereed Publications

Abstracts

Presentations


**Memberships in Scientific Societies**

Society of Environmental Toxicology and Chemistry (June, 2005-present)
Virginia Academy of Science (March, 2005-present)
Sigma Xi, the Scientific Research Society (April, 2004-present)
Beta Beta Beta National Biological Honor Society (Fall, 2000-present)