URIDINEDIPHOSPHATE-GLUCURONOSYLTRANSFERASE (UDP-GT) ONTOGENY AND PCB EFFECTS IN GALLIFORM BIRDS

Ryan J. R. McCleary

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Dr. F. M. Anne McNabb, Chair
Dr. David Bevan
Dr. Alan Heath

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Abstract

Hepatic UDP-GTs are partly responsible for metabolism of the thyroid hormone, thyroxine (T₄), in mammals, but little is known of UDP-GT activity in birds. To determine the ontogenic pattern of UDP-GT activity in precocial birds, we measured activity in Japanese quail (\textit{Coturnix japonica}) liver at days 12 and 14 of the 16.5-day incubation, 3 perihatch stages and <1, 1, 4, 6, 7, 20 and 42 days posthatch. We used an enzymatic reaction with \textit{para}-nitrophenol (\textit{pNP}) as substrate that was validated for quail tissue. The pattern of UDP-GT development included low embryonic activity, increased activity beginning in the perihatch period, a peak in activity at day 4 posthatch and a return to lower activity levels from day 6 to adults. The profile of UDP-GT activity, in relation to the ontogeny of circulating T₄ and triiodothyronine (T₃) in quail, is consistent with UDP-GT playing a role in regulating circulating T₄ and with the perihatch peak in T₃ stimulating the posthatch peak in UDP-GT activity. To examine the effects of polychlorinated biphenyls (PCBs) on UDP-GT in developing precocial birds, we dosed chicken (\textit{Gallus domesticus}) eggs with concentrations of PCB 126 from 0 to 0.80 ng/g egg (in sunflower oil) prior to incubation. Tissues were sampled at day 20 of the 21-day incubation and assayed for plasma hormones and UDP-GT activity. Eggs also were dosed with 0 or 0.25 ng PCB 126/g egg or with 0 or 0.64 ng/g egg of the coplanar PCB 77, allowed to hatch, and sampled at 42 days posthatch. There was no consistent pattern of altered thyroid hormones or UDP-GT activity in developing chickens exposed to either of these coplanar PCBs although previous studies indicated developmental alterations from exposure to the higher doses.

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Chapter 1: Literature Review

Pollutant Chemicals in Natural Environments

Due to previous widespread use of halogenated chemicals, including polychlorinated dibenzo-\(p\)-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), polychlorinated biphenyls (PCBs) and pesticides such as DDT, by industry, manufacturing and agriculture, many of these chemicals are now ubiquitous pollutants in natural environments.

Polyhalogenated aromatic hydrocarbons (PHAHs) have had some drastic effects on wildlife (Carson 1962) and continue to exert other, non-lethal effects (Colborn \textit{et al.} 1996) because of their persistence. Although lethal chemical effects have historically been the focus of toxicological studies, non-lethal effects, which are more difficult to quantify in terms of their damage to individuals, populations and species, are receiving much current attention.

One particularly disturbing category of non-lethal effects of some pollutant chemicals is their ability to disrupt endocrine control. This disruption may occur because the chemical mimics or blocks hormone action on target tissues (\textit{i.e.} it acts as an agonist or antagonist, respectively, and binds to receptors) or because the chemical alters the production or metabolism of hormones. Colborn \textit{et al.} (1996) chronicled some of the known problems related to endocrine disruption in natural animal populations, and McLachlan and Arnold (1996) reviewed evidence showing that some pollutants can act as estrogenic hormones. An important example of reproductive endocrine disruption can be seen in American alligators (\textit{Alligator mississippiensis}), from polluted lakes in Florida, that have altered concentrations of circulating steroid hormones (Crain \textit{et al.} 1998). Indeed, pollutant chemicals have been shown to produce sex reversals in alligators and turtles from these polluted lakes, thereby affecting population sex ratios (Guillette and Crain 1996).

Alterations in thyroid function, especially during ontogeny, can interfere with the normal development of many other, non-thyroidal systems in vertebrates. Low thyroid hormone (TH) concentrations during development can lead to slowed skeletal growth and maturation, and problems with muscle and central nervous system development (McNabb and King 1993). In amphibians, THs play crucial roles in the control of metamorphosis, so alterations in TH during development can result in abnormalities in the nature and timing of morphological and physiological development.

Descriptions of PCBs

PCBs, the chemicals of concern in this review, are widespread pollutants generated from many industrial processes. They have historically been used for many purposes, including as dielectric fluids in capacitors and transformers, as heat transfer fluids, as plasticizers in paints, in adhesives, as organic diluents, in laminating agents and in waxes (Bastomsky 1974; Safe 1990). Because of their widespread use and their
persistence, PCBs are ubiquitous. Because of their lipophilic nature, PCBs are stored in adipose tissue, and their bioaccumulation is magnified up the food chain. The presence of PCBs in animal tissues has been correlated with developmental and reproductive abnormalities in animals (see section on General Toxicology and Endocrine Disruption).

The basic structure of a PCB is two phenyl rings connected by a covalent bond. Each phenyl ring has five positions (numbered 2-6 and 2’-6’—Figure 1) where chlorines can potentially substitute in place of hydrogen atoms for bonding to carbons in the ring structure. On each ring, there are two ortho positions (those closest to the shared covalent bond between the rings), two meta positions (those adjacent to the ortho positions, but distal to the shared bond) and one para position (located most distal from the shared bond—see Figure 2). The naming of PCBs is based on the presence and location of the chlorine atoms on the phenyl rings (see Table 1). When chlorine atoms are absent from the ortho positions, the phenyl rings can rotate about the shared bond, due to the lower steric hindrance associated with hydrogen atoms than with chlorine atoms. In this conformation, PCBs have a higher probability of being in a planar position, one where both phenyl rings are in the same geometric plane. The steric hindrance of ortho chlorine atoms, however, reduces the probability of planarity (De Voogt et al. 1993).

Those PCBs with no ortho-substituted chlorines, but with both para and at least one meta position with chlorines, have the highest probability of being in a planar conformation. These coplanar PCBs (PCBs 77, 81, 126 and 169—Figure 3) have the highest toxicity when compared with other PCBs (Safe 1990). This is perhaps due to their chemical resemblance to the planar 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD—Figure 4), the most toxic of the PHAHs. TCDD binds to the aryl hydrocarbon receptor (AhR), which mediates its toxic response (Whitlock et al. 1996), and coplanar PCBs are thought to act at least partially through this mechanism. Safe (1990) proposed the use of toxic equivalency factors (TEFs), which express the relative toxicity of other PHAHs in terms of TCDD and found the coplanar PCBs (specifically PCB 126) to have the highest toxicity among PCB congeners.

**General Toxicology and Endocrine Disruption**

The exposure and some effects of pollutant chemicals on birds have been studied for years in Great Lakes herring gulls (*Larus argentatus*). In the 1970s, many abnormalities were noticed in gulls from colonies at heavily polluted sites. These included lower nest attentiveness, less nest defense, lower fecundity and lower embryo survival. These Great Lakes sites had some of the highest organochlorine pollutant loads of any areas tested (Peakall et al. 1978). Rattner et al. (1984) reviewed some of the known effects of pollutant chemicals on avian endocrine systems, including gonadal and thyroidal systems.

The herring gull has been considered a particularly good sentinel species for monitoring the overall health of an ecosystem because it is a top predator that consumes primarily fish and thus bioaccumulates persistent, lipophilic, organic pollutants. Gulls
nest colonially (so are relatively easy to monitor), have a wide geographic distribution (making it relatively easy to compare different sites with different pollutant loads), and adults generally remain on one territory year-round (Mineau et al. 1984).

The effects of organochlorine pollutants on thyroid gland size and histology were assessed in Great Lakes herring gulls from 1977 (shortly after PCB manufacture was outlawed) to 1982. A majority of the animals sampled were found to have goiter, and thyroid histopathology was highly correlated with the presence of environmental PHAHs (Moccia et al. 1986). Although the hatching success of these gulls has increased and reproductive and developmental toxicities have decreased over the past 25-year period of monitoring, the continued presence of high concentrations of organochlorines is sufficient to cause or exacerbate other physiological problems (Fox 1993; Peakall and Fox 1987). For example, a more recent study (Grasman et al. 1996) showed an association between environmental pollutant loads and immunological dysfunction in herring gulls and Caspian terns (Sterna caspia).

The possibility of organochlorine pollutant effects on birds in western Europe also has been studied and several lines of evidence are suggestive of thyroid alterations at the most polluted sites in the Netherlands and Belgium (Brouwer et al. 1998 and see section below on Effects of PCBs, Birds).

**General Background**

**Vertebrate Thyroid Function:**

Thyroid hormones are critical in the regulation of normal growth and development throughout the vertebrate classes and are involved in the regulation of metabolism in birds and mammals (McNabb and King 1993). Thus, alterations in circulating TH concentrations can have drastic permanent consequences for developing organisms.

Thyroid hormones [thyroxine (T4) and triiodothyronine (T3)] are produced by the iodination of tyrosine residues within thyroglobulin by thyroid follicle cells and are stored in colloid in the center of each thyroid follicle. For hormone mobilization, colloid enters follicular cells by endocytosis, the thyroglobulin is cleaved by lysosomal proteases and THs are released into adjacent capillaries by diffusion. The major TH produced and released is T4 (see Figure 5 for structure). T4 (3,3’,5,5’-tetraiodothyronine) is considered a prohormone because it can be converted to T3 (3,3’,5-triiodothyronine) by 5’ deiodination. T3, the thyroid hormone for which thyroid receptors have highest binding affinity, has higher biological potency than T4 and is produced primarily from the deiodination of T4 to T3 in extrathyroidal tissues. Both hormones, which are relatively lipophilic (hydrophobic), are transported in the blood primarily bound to carrier proteins (McNabb 1992).

Depending on the species, there are three major carrier proteins: thyroxine-binding globulin (TBG, found only in some mammals), transthyretin (TTR) and albumin.
In birds, TTR is the highest affinity T₄ carrier protein. In general, bound THs cannot enter cells and exert biological activity, but are important reservoirs of hormone for release to the small free hormone pool. Free THs can enter cells and produce physiological responses by binding to nuclear T₃ receptors. However, there is some evidence that TTR is involved in T₄ transport in at least one case, namely across the blood-brain barrier into the central nervous system (Robbins 1996). TH availability within extrathyroidal tissues also is influenced by deiodinases, which can alter the concentrations of T₃ by the deiodination of T₄ (Type I and II; outer-ring or 5’-deiodination) or by the degradation of T₄ to reverse T₃ (3,5,5’-triiodothyronine), which has no known biological activity (Type III; inner-ring or 5-deiodination; Leonard and Visser 1986; Leonard and Koehrle 1996).

In adult vertebrates, concentrations of T₄ are maintained homeostatically by a balance involving production in the thyroid gland and extrathyroidal deiodinations and degradation/excretion. A major mechanism of T₄ metabolism is conjugation, in which another molecule is added to T₄ to facilitate its excretion. For example, hepatic uridinediphosphate-glucuronosyltransferases (UDP-GTs—an important pathway altered by PCBs) make T₄ more water-soluble by glucuronidating it, thus facilitating its excretion in bile (Visser 1990; also see Biometabolic Enzymes section below).

Overall regulation of euthyroid TH concentrations is by the hypothalamic-pituitary-thyroid (HPT—Figure 6) axis. When TH concentrations in the blood are low, negative feedback is exerted on the anterior pituitary, which increases the output of thyroid stimulating hormone (TSH). Elevated TSH levels cause a subsequent increase in the production and release of THs from the thyroid, thereby increasing circulating TH concentrations (McNabb 1992).

**Avian Thyroid Development:**

Avian developmental patterns range from chicks that are fairly independent at the time of hatching (precocial) to those that are completely dependent on parental care after hatching (altricial). Precocial birds, like domestic chickens and Japanese quail, have their eyes open, are covered with down feathers, are capable of locomotion and self-feeding at hatch, show metabolic responses to cooling starting from time of hatching. In contrast, altricial birds like pigeons, sparrows and starlings, have closed eyes, lack down, are incapable of locomotion and are completely dependent on parental care for some period after hatch (Starck and Ricklefs 1998).

The patterns of thyroid development differ in these two modes of ontogeny (McNabb *et al.* 1998). In precocial birds, which show considerable maturation of their anatomical and physiological systems by the time of hatching, and which initiate thermoregulatory responses at hatch, most thyroid gland maturation occurs during the latter part of embryonic life and circulating concentrations of both THs have large peaks during the perihatch period. After hatching, T₄ concentrations decline, and then increase slightly to reach adult concentrations during early juvenile life. T₃ concentrations also decline posthatch then change little during juvenile life (McNabb and Hughes 1983).
Altricial birds, which are hatched at a less developed stage and do not show metabolic responses to cooling until some time (usually 1-3 weeks) after hatch, have relatively immature thyroid function at hatch. Their thyroid function increases posthatch and stabilizes at about the time when they develop thermoregulation (McNabb and Cheng 1985). These different patterns of avian thyroid development have implications for the time during which chemical pollutants are likely to alter thyroid function and cause other downstream effects on morphology and physiological development.

**Biometabolic Enzymes:**

Some endogenous and many exogenous chemicals are partly metabolized prior to excretion in vertebrates by Phase I and II hepatic biotransformation enzymes. These enzymes are important in the context of this project in two ways: (1) because chemicals like PCBs typically induce these enzymes, and (2) because some of these enzymes are involved in thyroid hormone regulation and turnover. Phase I and II biotransformation enzymes are categorized by their functions. Phase I cytochrome P450 enzymes generally catalyze degradative reactions (oxidation, reduction or hydrolysis) resulting in products with a polar functional group. Substances with exposed functional groups are more water-soluble, may be excreted in urine and are subject to conjugation by Phase II biometabolic enzymes. Phase II enzymes catalyze reactions that are synthetic, i.e. they attach a chemical moiety to the substrate. This results in a water-soluble product that is excreted more readily than the original substrate (Tephly and Burchell 1990; O’Flaherty 2000).

Two widely used assays of Phase I activity examine the activity of the enzymes ethoxyresorufin O-deethylase (EROD) and pentoxyresorufin-O-deethylase (PROD). Although these enzymatic processes usually detoxify toxic compounds, in a few cases P450s render compounds more fat-soluble and/or more toxic (Klasson-Wehler et al. 1989).

Phase II biometabolic enzymes, which are mostly found in the liver [but also occur in the skin, lungs, small intestine (rats—Li and Hansen 1997) spleen and head kidney (carp—Tayesse et al. 1998)], catalyze synthetic reactions (by adding a molecule that is covalently bound, i.e. conjugated to the substrate), such as attaching a glucuronic acid (for UDP-GTs) or glutathione [for glutathione S-transferase (GST)] to many potentially harmful exogenous chemicals. These reactions also are part of the pathway for the excretion of steroid and thyroid hormones that are relatively lipophilic. This enzymatic action makes relatively lipophilic compounds more water-soluble (hydrophilic), which increases their excretion in bile and urine. Although all UDP-GTs utilize endogenous uridine 5’-diphosphogluconic acid (UDPGA) as a reaction cofactor, each UDP-GT isozyme reacts with different substrates to different degrees. Basically, each isozyme has a range of compatible substrates and many isozymes may overlap activity for a specific substance. UDP-GTs have traditionally been grouped into categories based on either the substances they glucuronidate or the types of chemicals that induce their activity (Burchell and Coughtrie 1989; Mulder 1992).
UDP-GTs are separated into two large isozyme groups, depending on their substrates. UDP-GT1s are considered drug-glucuronidating (although their activity is not strictly limited to clinical compound substrates) and UDP-GT2s are considered steroid-glucuronidating (Emi et al. 1996). UDP-GT1A and 2A isozymes are considered active toward aromatic compounds and UDP-GT1B and 2B isozymes are considered active toward bilirubin. Thus, in this scheme, UDPGT1A would be considered a drug-glucuronidating enzyme with activity toward aromatic compounds.

The gene containing information for transcription of UDP-GTs has a variable exon 1 region followed by 4 conserved exons. Each exon 1 codes for the N-terminus of the subsequently translated protein and gives the UDP-GT its specificity (Masmoudi et al. 1997). Humans have at least 13 different UDP-GT1s (Masmoudi et al. 1997), and rats have at least 12 (Metz and Ritter 1998); the total number of UDP-GTs per species is continually being revised as more are discovered. While the functions of many individual isozymes have been determined in humans, some have not shown activity toward any tested substrate (Beaulieu et al. 1998). Currently, there is no published information on activities of individual UDP-GT isozymes in birds.

Effects of PCBs

Mammals:

The effects of PCBs on endocrine systems in mammals have been extensively investigated for one animal model, laboratory rats. Studies utilizing laboratory rats have shown significant effects on thyroid function in rats exposed to PCBs. In general, in this model, PCBs produce hypothyroid conditions by increasing TH excretion as a result of increased glucuronidation and the displacement of T4 from TTR binding.

Bastomsky (1974) analyzed the effects of the PCB mixture Aroclor 1254 on biliary excretion of T4 in male hooded rats. Using radioiodine (\(^{131}\)I) and labeled T4 (\(^{125}\)I-T4) administered to rats, he found that Aroclor 1254 treatment increased the thyroidal uptake of \(^{131}\)I, the biliary excretion of \(^{125}\)I-T4, and the proportion of \(^{125}\)I-T4 that was present in bile in the glucuronidated form. These results indicate that UDP-GTs were induced by PCBs, leading to increased glucuronidation and excretion of T4. The increase in \(^{131}\)I uptake suggests that the thyroid gland was stimulated by HPT axis activation through negative feedback by decreased circulating THs to compensate for the excreted T4. Further, this study showed decreased binding of THs to binding proteins, which also may have increased T4 excretion. The ability of some PCBs to displace T4 from TTR has been demonstrated in a number of \textit{in vitro} studies (e.g. McKinney and Waller 1994; Cheek et al. 1999), but the importance of this displacement in the regulation of thyroid function has not been adequately investigated.

Barter and Klaassen (1992b) characterized the types of hepatic UDP-GT in laboratory rats. They showed that hepatic glucuronidation of T4 was increased in rats dosed with Aroclor 1254, and that these rats experienced a 70-75% reduction in total and
free serum T₄ (Barter and Klaassen 1992a). By using thyroidectomized rats infused with TH solutions, they were able to demonstrate that these Aroclor 1254-induced reductions in circulating T₄ were due to an extrathyroidal mechanism i.e. the enhancement of T₄ excretion presumably due to the induction of hepatic UDP-GT. Similar results were obtained in a study in which thyroidectomized and T₄-supplemented rats were dosed with TCDD and showed increased induction of T₄-specific UDP-GT accompanied by decreases in plasma total and free T₄ concentrations (Schuur et al. 1997). Likewise, these results are consistent with the idea that it is the coplanar, TCDD-like congeners that are primarily responsible for the alterations in thyroid function associated with PCB exposure. Barter and Klaassen (1994) noted increased UDP-GT activity toward T₄ as well as reductions in total and free T₄ of 80-90% when rats were dosed with Aroclor 1254. Dosing with Aroclor 1254 also led to a 40% increase in TSH concentration, a 30% increase in thyroid gland weight and a 100% increase in ¹³¹I uptake, all changes indicative of feedback effects on the HPT axis and TSH effects on the thyroid gland.

Pregnant Sprague-Dawley rats exposed to coplanar PCB 77 showed high perinatal mortality of embryos (Harris and Bradshaw 1984). In the surviving offspring, many alterations in liver morphology were noted as well as an increase in the induction of hepatic UDP-GTs. Treated animals showed enzyme activity three times as high as in untreated controls. Weanlings of female Sprague-Dawley rats exposed to PCB 77 or TCDD in utero showed significantly decreased concentrations of plasma total T₄ (Seo et al. 1995). These rats, as well as those dosed with PCB 126, showed increases in UDP-GT activity toward the phenolic test substrate, para-nitrophenol (pNP) as well as accompanying increases in EROD activity. An examination of normal UDP-GT activity in adult Wistar rats found that females have significantly lower activities than males (Damanhouri and Tayeb 1994). Henry and Gasiewicz (1987) studied the effects of TCDD on TH concentrations and UDP-GT activity in adult male Sprague-Dawley rats and found significant decreases in T₄, significant increases in UDP-GT activity toward T₄ and, surprisingly, no change in TSH. This last part is significant because the decrease in T₄ does not bring a concomitant increase in TSH, as would normally be expected (see also Hood et al. 1999). Despite the rather consistent picture of PCB induction of hepatic UDP-GT in the studies above, one study showed the converse, namely decreases in UDP-GT activity in juvenile male Wistar rats exposed to either PCB 77 or 126, with 126 showing the greatest effect (Koga et al. 1994).

Although most of the effects of PCBs are attributed to coplanar (non-ortho) congeners, the mono-ortho congener 2,3,3’,4,4’,5-hexachlorobiphenyl (PCB 156) increases the activity of many liver enzymes, including Phase I enzymes P4501A1 and 1A2, as well as liver size and pathology in female Sprague-Dawley rats (Haag-Grönlund et al. 1997). Unfortunately this study did not specifically analyze the effects of these changes on thyroid function.

PCBs and PCB mixtures have been shown to cause alterations in circulating TH concentrations in both adult rats and the pups of PCB-exposed mothers. When pregnant female Long-Evans rats were treated with Aroclor 1254 (0, 1, 4 and 10 mg/kg) from day 6 of gestation through day 21 post partum, offspring of animals treated with the highest
doses had reduced circulating total T4 and T3 (TT4 and TT3) and free T4 and T3 (fT4 and fT3) concentrations by 21 days after birth, and some hormone concentrations were reduced earlier (Goldey et al. 1995). Weanling Sprague-Dawley rats exposed to coplanar PCB 77 (10, 100, 1000 or 10,000 ppb) or mono-ortho PCB 28 (2,4,4'-trichlorobiphenyl; at 50, 500, 5000 or 50,000 ppb) in their diets for 90 days had altered circulating T4 concentrations (Desaulniers et al. 1997). Consistent with most other studies of coplanar PCBs, circulating T4 was decreased in high-dose females exposed to PCB 77. With mono-ortho PCB 28, circulating T4 also was decreased in females receiving the highest dose. An unexpected result was that T4 was significantly decreased in both low-dose females and high-dose males receiving PCB 77. In the same study, adult male Sprague-Dawley rats dosed twice with coplanar 126 (6.25, 25, 100 or 400 µg/kg) had significantly reduced T4 in the three groups with the highest doses and reduced T3 in the groups with the two highest doses.

**Birds:**

In laboratory studies, chickens have been the main models used to elucidate the effects of pollutant chemicals in birds. In terms of embryonic mortality and Phase I biometabolic enzyme induction (measured as EROD activity), there is a hierarchy of declining effects of PCB congeners in the following order: 126 > 77 > 169 > 105 (Brunström 1990). PCBs 126, 77 and 169 are coplanar, or dioxin-like, congeners and PCB 105 is a mono-ortho substituted congener that has some properties similar to the coplanar congeners (Kannan et al. 1988). PCB 126 caused 90% mortality in day 10 embryos dosed at day 7 of incubation with a relatively low concentration of 2 ppb. Brunström and Andersson (1988) showed a similar hierarchy in terms of LD50, with PCB 126 being most toxic, followed by PCBs 77 and 169 (LD50s of 3.1, 8.6 and 170 ppb, respectively). Powell et al. (1996) injected PCB 126 in triolein into yolks and saw a dose-dependent increase in developmental abnormalities and mortality. They also found that doubling the dose from 1.6 ppb to 3.2 ppb increased mortality from 21.7% to 91.7%. Lorenzen et al. (1997) showed in vitro induction of EROD in chicken embryo hepatocytes by PCB 126 in a dose-dependent manner. The site of chemical injection into eggs can influence effects on embryos as illustrated by the work of Henshel et al. (1997) that indicated TCDD injected into the yolk of chicken eggs had a greater toxic effect (in terms of mortality) than when injected into the air cell.

One study used embryos of chicken eggs dosed with different PCB congeners (PCBs 54, 77 or 80) or mixtures (Aroclor 1242 or 1254) prior to incubation and killed on the final day of the 21-day incubation period (Gould et al. 1999). Embryos with the highest doses of either mixture (100 ng/egg or ~6.7 ppb in yolk) showed significant decreases in plasma T4 concentration. Embryos dosed with the highest concentration of Aroclor 1242 also exhibited a significant decrease in plasma T3.

Galliform species other than chickens also have been manipulated in the laboratory to determine the effects of pollutant chemicals. Riviere et al. (1978) used Japanese quail (Coturnix japonica) to study the effects of Phenochlor, a PCB mixture with about 50% chlorine, on enzyme induction in treated adults and their offspring.
Dosed adult animals showed significant increases in liver weight, cytochrome P450 concentrations, and several enzyme activities, including UDP-GT activity toward pNP.

Most studies on wild birds have been done on animals that have been exposed to PCBs in their natural environments, although in some cases wild-caught animals have been treated with PCBs in the laboratory. Murk et al. (1994b) injected one intraperitoneal dose of either PCB 77 or Clophen A50, a PCB mixture, into 28-day old wild-caught eider ducklings (Somateria mollissima) and found significant increases in EROD activity 10 days after injection. There were significant correlations between high body lipid PCB concentrations and decreases in the rates of body weight gain and beak length growth in the Clophen A50-dosed groups, increased relative liver weight and EROD activity in both PCB 77 and Clophen-dosed groups, and reduced plasma T4 and T3 and hepatic retinoid concentrations in PCB 77-dosed groups.

Female mallard ducks (Anas platyrhynchos) have shown rapid bioaccumulation of organochlorine pollutants when maintained at a sewage plant (Custer et al. 1996). These birds showed a linear increase in PCB concentration with time, and P450 enzyme activity increased proportionately with PCB loads. Adult male mallard ducks, gavaged twice weekly for five weeks with different concentrations of Aroclor 1254 (0, 4, 20, 100, 250 or 500 mg/kg), showed significant EROD and PROD induction at 20 mg/kg and higher, with the greatest induction in those dosed at 100-500 mg/kg. Relative liver and thyroid weights in these birds were increased at the higher doses, while plasma total T3 showed a dose-dependent decrease (Fowles et al. 1997). It is unfortunate that this study did not analyze Aroclor 1254 effects on T4 because the enhanced excretion expected to result from PCB treatment is specific to T4, and the effects on T3 are much more complex.

The effects of some pollutant chemicals on cormorant embryos also have been studied. Powell et al. (1998) injected eggs of double-crested cormorants (Phalacrocorax auritus) with PCB 126 (0, 70, 175, 349 or 698 µg/kg egg) or TCDD (0, 1.3, 5.4, 10.7 or 21.4 µg/kg egg) prior to incubation and sampled the chicks just after hatching. They found increased hepatic EROD activity in all dosed groups. The LD50 of TCDD was about 44.25 times lower than the LD50 for PCB 126.

A further study used artificially incubated eggs of the common tern (Sterna hirundo) collected from a natural habitat in the Netherlands (Bosveld et al. 2000). Hatchlings were fed fish with different concentrations of coplanar PCB 126 alone or in conjunction with non-coplanar PCB 153 for 21 days posthatch. There was a statistically significant negative relationship between concentrations of coplanar PCBs (including PCB 126) in liver and plasma T4 concentrations.

**Organochlorine Pollutant Effects on Avian Liver Biotransformation Enzymes:**

Birds from habitats highly contaminated with organochlorine pollutants typically have significant increases in activity of hepatic Phase I biotransformation enzymes such as EROD. However, there is little basic knowledge about Phase II biotransformation enzymes that are assumed to be important in thyroid homeostasis in birds.
There are posthatching developmental increases in liver UDP-GT activity in chickens. Coulet et al. (1996) examined changes in UDP-GT activity at different ages in male Sasso broiler chickens ranging in age from 3 to 12 weeks. UDP-GT steadily increased over the time of the study, with activities at 9 and 12 weeks being significantly higher than at 3 weeks. However, embryonic development of UDP-GT has not been investigated, although most thyroid maturation occurs during embryonic life in these precocial birds. A comparative study of hepatic UDP-GT activity in 10-month old ostriches (Struthio camelus), adult white Leghorn chickens of both sexes and 12-week old male Sprague-Dawley rats showed that ostriches had much lower activity per gram of liver than chickens, and the activity in both avian species was significantly lower than in the rats (Amsallem-Holtzman and Ben-Zvi 1997). No studies have addressed the role of UDP-GT in thyroid hormone homeostatis or the importance of UDP-GT in altering thyroid state in birds. However, one study (Murk et al. 1994a) did attempt to evaluate the importance of UDP-GT alterations in PCB-exposed birds (see below).

Phase I enzymes (immunodetected P4501A i.e. CYP1A) appear to be induced by environmental PCBs in pipping embryos of black-crowned night herons (Nycticorax nycticorax) in the United States. Samples taken from three polluted sites in Wisconsin and California exhibited significant increases in Phase I enzymes compared to the Virginia reference site, and Phase I enzyme activity was correlated with total concentrations of PCBs and 11 specific congeners thought to exert toxicity through the aryl hydrocarbon receptors (AhR; Rattner et al. 1994).

A study of common tern embryos in western Europe found no differences in Phase II UDP-GT activity toward T4 and mean site PHAHs in yolk sacs (Murk et al. 1994a). Although this study did find strong correlations between UDP-GT activity and Phase I (EROD) enzyme activity, it is difficult to interpret because circulating thyroid hormones were not altered in relation to site PCBs. Unfortunately, this study did not include validation of the enzyme assay techniques for use on avian tissues.

In general, the literature on PCB effects on thyroid status in birds is limited, and the results of different studies often are inconsistent with each other. Specifically, the relationships between environmental PCB load, induction of Phase II liver biotransformation enzymes and alterations of thyroid hormones are unclear. To understand PCB effects on thyroid function in birds, future studies must examine the normal ontogeny of UDP-GT activity and the effects of pollutant chemicals on UDP-GT activity and thyroid status in the wild and in a laboratory setting.
Chapter 2: Introduction

The presence of high concentrations of pollutant chemicals, including polychlorinated biphenyls (PCBs), in natural environments has traditionally been of great concern because of their toxicity. Since the production of PCBs was outlawed in 1976, the concentrations found in wildlife and natural environments have declined (Peakall and Fox 1987; Fox 1993). However, the persistence of such chemicals is of concern not only in terms of acute toxicity, but also in terms of their potential for endocrine disruption.

It has been suggested that PCBs may be causing disruption of thyroid function in wild birds (Fox 1993). Herring gulls (Larus argentatus) exposed to pollutant loads in the Great Lakes have exhibited increased ontogenic and immunological problems compared to reference populations (Peakall et al. 1978; Moccia et al. 1986; Grasman et al. 1996). Thyroid gland histopathology in gulls with high environmental PCB exposure (Moccia et al. 1986) and developmental effects in chicken embryos exposed to PCBs in ovo (Powell et al. 1996) are suggestive of thyroid abnormalities. Herring gulls are considered important indicators of habitat quality because they are piscivorous top predators that are exposed to concentrated pollutant loads through bioaccumulation in the food chain. Recent work in our laboratory has demonstrated that Herring gulls from highly polluted PCB sites in the Great Lakes have significantly depleted thyroid hormone stores in their thyroid glands compared to gulls from reference sites (McNabb et al. 2001).

Effects of PCBs on thyroid physiology have been studied mainly using laboratory rats as models, and the thyroid alterations observed in these studies serve as a frame of reference for studies in birds. PCBs are known to disrupt thyroid function in rats by indirect mechanisms such as induction of hepatic Phase II biotransformation enzymes that increases T4 conjugation and excretion in bile and displacement of T4 from binding proteins that presumably also increases T4 excretion (Brouwer et al. 1998). If enhanced T4 excretion exceeds the capacity of the thyroid gland to produce T4, despite HPT axis stimulation, the resulting hypothyroidism would be expected to have serious consequences for organismal development (McNabb 1992).

Despite the relatively well-developed picture of PCB effects on thyroid function in laboratory studies of rats, it is not clear whether birds are affected in the same ways. Studies of PCB effects on birds have yielded conflicting results about whether circulating thyroid hormones (both total and free) are altered and whether there is thyroid gland hypertrophy indicative of HPT axis activation (reviewed in Dawson 2000). Common tern (Sterna hirundo) chicks from PCB-polluted sites along the Rhine and Meuse rivers in the Netherlands and Belgium did not show an inverse relationship between colony PCBs and plasma THs (Murk et al. 1994a). Likewise, van den Berg et al. (1994) found no significant concentration-effect relationship between yolk sac PCBs and plasma THs in hatchling cormorants. These results were surprising because they found significantly decreased plasma TT4, TT3 and fT4 in the colony of cormorants from the PCB-polluted site compared to the reference colony.
The few studies of PCB induction of liver enzymes, that are presumed to facilitate T₄ excretion in birds, also have yielded inconsistent results. Female Japanese quail (Coturnix japonica) dosed with a PCB mixture showed an increase in UDP-GT activity (Riviere et al. 1978), but chicks from laboratory-incubated common tern (Sterna hirundo) eggs collected at environmentally polluted sites showed no significant differences in hepatic UDP-GT activity compared to a reference site (Murk et al. 1994a). However, hepatic Phase I biometabolic enzyme activity was correlated with PCB concentrations in individual terns from these sites (Bosveld et al. 1995).

Because we are concerned with the disruptive effects of PCBs on avian thyroid systems, we analyzed induction of activity of the Phase II biotransformation enzyme complex known as uridinediphosphate-glucuronosyltransferases (UDP-GTs). UDP-GTs are partially responsible for the removal of T₄ from the circulation in vertebrates, and are induced in laboratory rats exposed to the PCB mixture, Aroclor 1254 (Barter and Klaassen 1992a, 1994). Such induction, which leads to conjugation of xenobiotics, was found to not only affect the pollutant chemical, but also to increase the biliary clearance of glucuronidated T₄ (Bastomsky 1974), thereby decreasing circulating T₄ concentrations (Barter and Klaassen 1992a, 1994).

Although wild bird populations are normally exposed to mixtures of chemical pollutants, it is important to determine which components of mixtures are responsible for observed effects. This paper reports laboratory studies, conducted in galliform birds, that were designed to determine how PCBs might affect the key liver enzymes known to conjugate T₄ and facilitate its excretion in mammals. Our first objective was to investigate the developmental pattern of hepatic UDP-GT in embryonic and early posthatch life in Japanese quail (which have an abbreviated but otherwise identical pattern of thyroid development to that in chickens; McNabb 1988). This study is important for understanding of the role of this enzyme in the pattern of avian thyroid development and aids in the interpretation of the second objective, which included studying the effects of PCBs at a single developmental stage.

The second objective of this study was to determine the effects of the PCB congener 3,3′,4,4′,5-pentachlorobiphenyl (PCB 126) on hepatic UDP-GT in chicken embryos. PCB 126 is a co-planar, or dioxin-like, congener (Haag-Grönlund et al. 1997) that acts at least partially through the aryl hydrocarbon receptor (AhR) and is considered the most toxic of the PCBs (Brunström 1990; Safe 1990; Whitlock et al. 1996). PCB 126 is one of the most common PCBs (in terms of presence and concentration in natural environments; Safe 1990). We also report preliminary data on the effects of 3,3′,4,4′-tetrachlorobiphenyl (PCB 77, also a co-planar congener) on UDP-GT activity in chicken embryos.
Chapter 3: Materials and Methods

Animals

Fertile eggs were obtained from a random-bred Japanese quail (Coturnix japonica) colony maintained in our laboratory at Virginia Tech. Quail eggs were incubated in a Humidaire Hatchette (incubator/hatcher) at a temperature of 38°C and 90% relative humidity. Chicks were maintained in a brooder with a temperature gradient for the first three weeks posthatch and fed Sporting Bird Starter Diet (Southern States, Christiansburg, VA).

Fertile White Leghorn chicken (Gallus domesticus) eggs from a random bred/out bred colony were purchased from Truslow Farms for PCB experiments conducted in K. Grasman’s laboratory at Wright State University (WSU; Dayton, Oh). They were incubated at 37.5°C and 55% relative humidity until day 20 of the 21-day incubation period for the embryonic experiments. For the experiments on chicks, the eggs were placed in hatching trays in a horizontal position 3 days prior to the predicted hatching date. Chicks were allowed to hatch and dry before being moved to the animal care facility for maintenance and were maintained on chicken starter feed and water ad libitum until time of sampling.

Experimental Design

Quail UDP-GT Ontogeny:

For our study of the development of UDP-GT in a precocial bird (Japanese quail) we used embryos sampled on days 12, 14, and the perihatch period of the 16- to 17-day incubation period (N = 5-17 per developmental age). Perihatch embryos sampled on days 16 and 17 were separated into three stages: beak not into air cell (NI), internal pipping (i.e., beak into the air cell—I) and external pipping (i.e. beak pipped through the eggshell—S). Chicks were sacrificed by decapitation at <1, 1, 4, 6, 7 and 20 days posthatch. Adult quail also were sampled. Samples were frozen at –80°C until analysis. Livers were homogenized individually if they were 0.25 mg or larger, or pooled to yield a 0.33 mg or larger sample in the case of smaller embryos or chicks.

Effects of PCBs on Hepatic UDP-GT Activity in Developing Chickens:

To examine different aspects of UDP-GT activity on precocial birds, we performed three experiments. In Experiment 1, day 20 embryos from eggs dosed with PCB 126 were sampled to assess hepatic UDP-GT activity. Due to the constraints of sampling large numbers of eggs at one time, several sets of 10-17 eggs (runs) with different doses of PCB 126 were used. Because there were no statistically significant differences between runs, all runs were combined for the final analysis.

Chicken eggs were dosed with 3,3’,4,4’,5-pentachlorobiphenyl (PCB 126) and embryos and chicks were sampled in the laboratory of K. Grasman at WSU. PCB 126
was administered in sunflower oil onto the air cell of eggs prior to incubation. Doses included were 0 (sunflower oil vehicle), 0.05, 0.13, 0.32, 0.48, 0.64 or 0.80 ng PCB 126/g egg. Non-injected controls also were included. Eggs were incubated, and the embryos were sacrificed and sampled on day 20 of the 21-day incubation period. Livers were removed, divided into right and left lobes and stored at –80°C until analysis. Blood samples were collected in heparinized tubes and centrifuged, and separated plasma was stored frozen until analysis.

In Experiments 2 (embryonic exposure to PCB 126) and 3 (embryonic exposure to PCB 126 or 77), chicks from the PCB-dosed eggs were sampled at day 42 posthatch. For Exp. 2, eggs were dosed with PCB 126 at concentrations of 0, 0.25 and 0.50 ng/g egg weight. In Exp. 3, eggs were dosed with PCB 126 (0.25 ng/g egg weight), PCB 77 (3,3’,4,4’-tetrachlorobiphenyl—0.64 ng/g egg weight) or sunflower oil only. The doses of PCBs we used were designed to investigate PCB effects at levels with relatively low toxicity [note that the highest dose (0.8 ng/kg) of PCB 126 resulted in 40% mortality].

**Measurements of Hepatic UDP-GT Activity**

Tissues were examined for activity toward para-nitrophenol (pNP), a substrate utilized by all UDP-GT isozymes induced by phenolic compounds, and preliminary studies were done using labeled T4 as substrate.

**Tissue Preparations:**

Crude liver homogenate (HOM) was prepared by 10 strokes of a ground glass pestle in a 3 ml Kontes 21 glass homogenizer containing liver and buffer. Depending on the desired tissue dilution for different parts of the study, HOM was one part liver by weight plus two (3X) or four (5X) parts Tris-HCl buffer (50 mM Tris with 150 mM KCl) by volume. HOM samples were frozen, thawed and filtered through one layer of nylon organdy to remove congealed lipids and fragments of connective tissue, then was stored at -80°C.

For comparison with HOM preparations, microsomes (MIC) were prepared from HOM samples by differential centrifugation. First, HOM was centrifuged at 10,000g for 20 min at 0°C to obtain a supernatant “post-mitochondrial fraction” (PMF) which in turn was centrifuged at 125,000g for 60 min at 4°C in a Beckman TL-100 ultracentrifuge. After the supernatant was discarded, the microsomal pellet was resuspended in 0.25 M sucrose and stored at -80°C (Barter and Klaassen 1992a). There was considerable loss of UDP-GT activity in MIC preparations compared to HOM, relative to original tissue used so all data reported in this study were generated using HOM.

**Methods Validation:**

To measure liver UDP-GT in birds, we first needed to validate the use of a UDP-GT assay, previously used for mammalian and fish tissue, for use with avian liver preparations. Our validations determined the assay conditions under which enzyme
activity was proportional to both enzyme concentration and incubation time for each experiment. We used adult quail liver for the initial validation studies, and then checked for valid assay conditions with liver from the embryonic chicken and quail used in the different experiments.

**para-Nitrophenol Assay for UDP-GT Activity:**

Hepatic UDP-GT activity toward phenolic substrates was measured as decolorization of para-nitrophenol (pNP), by modification of the methods of G. A. Fox (Environment Canada; personal communication) and Castren and Oikari (1983). The decolorization of pNP from yellow to clear with conjugation was measured as absorbance at 400 nm. In this method, 50 µl of HOM or MIC was mixed with 100 µl of 10.0 mM uridine 5'-diphosphoglucuronic acid (UDPGA) solution in buffer-diluent (0.2 M Tris-HCl w/0.02% Triton X-100, pH 7.4). This mixture was vortexed and pre-incubated for 5 min at 37°C to conjugate any potentially confounding endogenous phenolic compounds and to bring the mixture to reaction temperature. Then, 50 µl of 5.0 mM pNP solution in buffer-diluent was added. The mixture was vortexed and incubated at 37°C for 30-180 min, depending on the tissue preparation (HOM or MIC) and on other factors, such as animal age and species, which might affect the enzyme content of the tissues. At the end of incubation, the reaction was stopped by adding 900 µl of ice-cold 3% trichloroacetic acid (TCA), and the tubes were vortexed and left on ice for 10 min. The tubes were then centrifuged on a Jouan MR 18 12 centrifuge for 20 min at 10,000g and 0°C. Then 900 µl of the supernatant was added to tubes containing 100 µl 5 N NaOH. This alkaline mixture was vortexed and a 500 µl aliquot of each sample was placed in a disposable 4.5 ml polystyrene cuvette, diluted with 2.0 ml deionized H2O and mixed by inversion. The absorbance of each sample was read on a Beckmann DU-640 spectrophotometer at 400 nm. Blanks were treated as above except that TCA was added immediately after the addition of pNP solution (zero time blanks). For each sample, we did triplicate tubes for enzyme activity and for zero time blanks. We tested several types of blanks (0 time blanks, incubated blanks that contained no tissue and incubated blanks that contained no UDPGA) and found no detectable difference in absorbance between the three types.

Standard conditions for pNP assays were as described above, but with the specifics that follow. The chicken embryo experiments were done with a 5X homogenate and a 180 min incubation time. The quail UDP-GT developmental study was done using 5X HOM for a 40 min incubation period and the 42-day posthatch chicken study was done using 12.5X HOM.

**Labeled T4 Assays for UDP-GT Activity:**

To compare the amount of enzyme activity indicated by the pNP assay to the amount of T4 glucuronidated, we investigated UDP-GT activity using labeled T4 as substrate by a modification/combination of techniques (Beetstra et al. 1991; Barter and Klaassen 1992a). Each incubation tube contained 50 µl tissue HOM or other preparation, 80 µl buffer (100 mM Tris-HCl w/13.33 mM MgCl2, 0.033% Brij-58, 1.87 mM saccharic acid-1,4-lactone, pH 7.4), 30 µl 33.33 mM UDPGA in buffer, 20 µl 1.0 mM unlabeled T4
in H$_2$O and 20 µl $^{125}$I-T$_4$ in buffer (~100,000 cpm). The concentration of the labeled T$_4$ was negligible (0.304 nM) and did not significantly increase the overall concentration of T$_4$ in the incubation mixture. The tubes were incubated in a 37°C water bath for 60 min, and the reaction was halted by the addition of 200 µl of ice-cold methanol (MeOH). This mixture was vortexed, then centrifuged for 15 min at 10,000 g and 0°C. Supernatant (350 µl) was then added to 700 µl 0.1 N HCl, vortexed, and loaded onto Sephadex LH-20 columns (1.0 ml bed volume) that were prewashed with 0.1 N HCl. The sample components were eluted in a step-wise manner with 6 X 1.0 ml aliquots of 0.1 N HCl followed by 7 X 1.0 ml aliquots of deionized H$_2$O and 4 X 1.0 ml aliquots of 0.1 N NaOH/ethanol (1:1, v/v). These elution fractions were collected and counted. As Figure 7 shows, there are three distinct peaks of radioactivity that correlate with the three elution solvents. Unbound $^{125}$I was eluted in the acid fraction, glucuronidated $^{125}$I-T$_4$ (T$_4$G) was eluted in the water fraction, and the unbound $^{125}$I-T$_4$ was eluted in the alkaline ethanol fraction (Beetstra et al. 1991; Finnson and Eales 1997).

For the pNP assay, Tris-HCl, Triton X-100, pNP, UDPGA and TCA were supplied by Sigma (St. Louis, MO); KCl and 4.5 ml disposable polystyrene cuvettes were supplied by Fisher Scientific (Pittsburgh, PA). For the labeled T$_4$ assay, high specific activity radiolabeled T$_4$ (1200 µCi/µg $^{125}$I-T$_4$) was supplied by New England Nuclear (Boston, MA); MgCl$_2$, HCl, MeOH and NaOH were supplied by Fisher Scientific (Pittsburgh, PA); Brij-58, saccharic acid-1,4-lactone and Sephadex LH-20 were supplied by Sigma (St. Louis, MO); and Poly-Prep chromatography columns were supplied by Bio-Rad Laboratories (Hercules, CA).

**Verification of Measurements of UDP-GT Activity Using Mammalian Tissue:**

To demonstrate that these techniques could determine differences in UDP-GT activity between control animals and those dosed with pollutant chemicals, C57Bl/6++ mice were used. These mice, which are sensitive to TCDD and exhibit relatively high background levels of UDP-GT activity, were dosed with either 50 µg/kg body weight TCDD in corn oil or only corn oil (as control) and their livers were prepared as described previously.

**Protein Determinations**

Protein determinations were done spectrophotometrically at 595 nm using the Pierce Coomassie Protein Assay (Pierce, Rockford, IL). For the protein assay, homogenate samples were diluted 300X and a 25 µl aliquot was added to 1.0 ml of Coomassie blue reagent, mixed and read against a standard curve (0-1000 ng/ml) from dilutions of the bovine serum albumin solution provided by Pierce. All protein measurements were done in triplicate.

**Statistics**

Quail UDP-GT ontogeny data were analyzed using a one-way ANOVA (SPSS; Chicago, IL) with Tukey’s HSD post hoc test. Differences in means were considered
significant at p < 0.05. Embryonic and 28-day chicken data were analyzed using 95% confidence intervals generated by SigmaPlot (SPSS). Mouse samples were analyzed by t-test for equality of means, equal variances not assumed (SPSS).
Chapter 4: Results

Validation Studies for pNP Assay of UDP-GT Activity

To calculate pNP glucuronidation activity, we determined the molar absorption coefficient for pNP solutions for our conditions, with different concentrations of pNP in buffer as the standard curve. From this standard curve (Figure 8), and using Beer’s Law ($\Delta A = \ell \Delta c \varepsilon$, where $\Delta A$ is change in absorbance, $\ell$ is length of light path, $\Delta c$ is change in solution concentration and $\varepsilon$ is the molar absorption coefficient), the molar absorption coefficient for pNP was determined to be 14,587 M$^{-1}$cm$^{-1}$.

For quail liver, UDP-GT activity was shown to be proportional to enzyme concentration using HOM preparations up to 2.6 µg HOM protein/ml in the 200 µl reaction mixture (Figure 9). Also, using 2.8 µg HOM protein/ml, enzymatic activity was linear for times up to 90 minutes (Figure 10). These findings led to standard assay conditions, using homogenate concentrations containing approximately 2.8 µg HOM protein/ml and an incubation time of 40 minutes for adult quail liver samples. For the developmental study of quail liver, an incubation time of 40 minutes was also used, but, due to differences in protein content at different ages, homogenate concentrations ranged from 2.9 to 8.1 µg HOM protein/ml in the 200 µl incubation.

The standard assay conditions using chicken liver HOM were altered based on developmental age of the tissue. In general, we found embryonic chicken tissue to be lower in protein content than adult tissue. Enzyme activity in juvenile chicken HOM preparations was linear with time up to 120 minutes using 4.7 µg HOM protein/ml reaction mixture (Figure 11). Assays on juvenile chicken liver were done using approximately 6.8 µg HOM protein/ml and 20 minute incubations; embryonic assays were done using approximately 11.0 µg HOM protein/ml at 180 minutes.

To demonstrate that the pNP assay could detect induction of UDP-GT, we used liver HOM of TCDD-dosed C57Bl/6++ mice. These mice were dosed with up to 50 µg/kg TCDD in a sunflower oil vehicle. The assay clearly detected induction of pNP UDP-GT in TCDD-dosed compared to control mice that received corn oil (Figure 12).

Quail UDP-GT Ontogeny

The profile of UDP-GT activity toward pNP during development in Japanese quail is shown in Figure 13. Embryonic liver UDP-GT activity levels increased slowly until the end of the perihatch period (24 hours posthatch). Then, UDP-GT activity increased markedly between days one (>24 hours) and day 4 posthatch then declined to levels slightly higher than those in embryos by day 6 posthatch. Enzyme activity did not change significantly between day 6 and adult ages (sexually mature birds of >6 weeks of age). When compared to day 12 or 14 embryos, significantly higher activity was found at days 1, 4 and 7 posthatch. When compared to adults, liver UDP-GT activity was significantly higher at day 4 posthatch (one-way ANOVA, Tukey’s HSD test, p < 0.0001).
Effects of PCBs on Hepatic UDP-GT Activity in Developing Chickens

Experiment 1. Embryonic Exposure to PCB 126/Embryos:

UDP-GT activity toward \( p \)NP was not significantly different from either vehicle-injected or non-injected controls in any of the groups dosed prior to incubation and sacrificed on day 20 of incubation. Whether all data were analyzed together (Figure 14) or as individual runs (8/98, 10/98 and 1/99, Figures 15, 16 and 17, respectively) there were no trends toward increasing activity (indicative of enzyme induction) with increasing PCB doses. Likewise, plasma T\(_4\) did not differ between either control (vehicle-injected or non-injected) and any of the dosed groups. Plasma T\(_3\) did not show any consistent pattern of significant differences in relation to PCB doses although T\(_3\) was significantly increased, compared to both types of controls, at doses of 0.0512 and 0.48 ng/g egg weight (Figure 18).

Experiment 2. Embryonic Exposure to PCB 126/Chicks:

In chicks dosed with PCB 126 prior to incubation and sacrificed at day 42 posthatch there were no significant differences in UDP-GT activity between control and PCB 126-injected groups (Figure 19).

Experiment 3. Embryonic Exposure to PCB 126 or 77/Chicks:

Neither PCB 126-dosed (0.25 ng/g egg) nor PCB 77-dosed (0.64 ng/g egg) groups showed significantly altered UDP-GT activities toward \( p \)NP compared to vehicle-injected controls. These tissues were from chicks dosed prior to incubation and sacrificed at day 42 posthatch (Figure 20).

T\(_4\) Assay

In preliminary experiments using the T\(_4\) assay, we were able to show separation between control and TCDD-dosed (50 \( \mu \)g/kg) mouse liver HOM (Figure 21). This indicates the potential for utilizing radiolabeled T\(_4\) as substrate to specifically examine UDP-GT activity in the context of thyroid function. In preliminary experiments with adult quail HOM, we have been able to show linearity and proportionality up to 90 minutes with 2.7 \( \mu \)g HOM protein/ml in the 200 \( \mu \)l reaction mixture (Figure 22).
Chapter 5: Discussion

The present study of UDP-GT activity in galliform birds was part of a larger project investigating PCB effects on avian thyroid development and function. Overall, this larger project addresses the effects of pollutant chemicals (including highly prevalent PCBs) on thyroid status in wild-caught herring gulls from the Great Lakes and the effects of specific PCB congeners on thyroid function in laboratory-reared chickens. Specifically, the present study investigated one hepatic enzyme system that is induced in some vertebrates by PCBs and is involved in the metabolism and excretion of T4.

There were two main aspects to this study: (1) the description of the pattern of UDP-GT ontogeny in a precocial galliform bird throughout embryonic and early posthatch life, and (2) the investigation of UDP-GT responses to coplanar PCB exposure during embryonic development.

**UDP-GT Ontogeny in Quail**

Because UDP-GT is responsible, in part, for normal metabolism of T4 (glucuronidation) resulting in increased hydrophilicity and consequent excretion in urine and bile, we were interested in comparing UDP-GT activity with T4 concentrations during development. To understand the regulation of circulating thyroid hormones at different times during development, it is necessary to understand the pattern of UDP-GT ontogeny as well as the development of thyroid hormone production and secretion (McNabb et al. 1998). To date, only one study (Coulet et al. 1996) has examined hepatic UDP-GT activity in a developing bird. That study, however, examined only four ages in juvenile chickens (3, 6, 9 and 12 weeks posthatch). The present study is, to our knowledge, the first investigation of UDP-GT during the period of embryonic and early posthatch life when most thyroid development occurs.

Circulating thyroid hormone concentrations reflect a balance between hormone production/release from the thyroid gland and thyroid hormone metabolism/excretion. During the second half of incubation in precocial birds, embryonic plasma T4 concentrations increase several-fold (McNabb et al. 1981). During the perihatch period, plasma T4 increases even more dramatically to peak at concentrations several-fold those in late incubation. This peak of circulating T4 is largely due to TSH signals from the pituitary as part of HPT axis maturation (Thommes 1987). The low UDP-GT activity we found during late incubation and the perihatch period are suggestive of low T4 metabolism and excretion until after hatch. This low UDP-GT activity, in conjunction with increased thyroid gland function, must contribute to the perihatch peak in T4. Just after hatch, plasma T4 concentrations decrease precipitously, probably partly due to the posthatch increase in UDP-GT found in the present study (Figure 23).

Studies of UDP-GT regulation in juvenile thyroidectomized rats (Masmoudi et al. 1996) may help explain the role of UDP-GT in the developmental pattern of circulating T4 in precocial birds. Masmoudi et al. (1996) found evidence that high T3 can induce hepatic UDP-GT activity in young rats. As shown in Figure 23, T3, like T4, peaks during
the perihatch period (in this case due to changes in deiodination activity; Freeman and McNabb 1991; Darras et al. 1992). If UDP-GT regulation in avian development is similar to that in rats, the perihatch peak in plasma T3 could be important in the induction of increased UDP-GT activity between days 1 and 4 posthatch in quail in the present study.

**Embryonic and Posthatch Exposure to PCB 126 in Chickens**

In previous studies, laboratory rats exposed to PCB mixtures, to coplanar PCB congeners and to TCDD typically had decreased circulating T4 concentrations and increased hepatic UDP-GT activity (Brouwer et al. 1998). Because UDP-GTs are thought to be involved in T4 metabolism/excretion in birds and because some PCBs have decreased circulating T4 in some avian studies (e.g. Eider ducklings, Murk et al. 1994b; chicken embryos, Gould et al. 1999) we hypothesized that avian embryos exposed to PCBs in the egg would have decreased plasma T4 and increased hepatic UDP-GT activity. PCB 126 was chosen as the first congener to investigate because it is similar structurally to TCDD (coplanar), it has high toxicity relative to other PCBs, it is present at high concentrations in many polluted environments and it is highly persistent. Also, PCB 126 exposure in embryonic chickens has been shown to cause developmental abnormalities, while in the same study TCDD exposure did not (Powell et al. 1996). Unfortunately, this study did not include information on TH concentrations so it is not clear whether altered thyroid function was the direct cause of these developmental abnormalities. In another study of embryonic chickens, TCDD induced the Phase I biotransformation enzyme ethoxyresorufin O-deethylase (EROD) but did not cause either developmental abnormalities or changes in TH levels (Janz and Bellward 1996). It should be noted that these authors did not measure UDP-GT. Murk et al. (1994a) did find a correlation between EROD and UDP-GT in the common tern (Sterna hirundo), but relatively little is known about the relationships between induction of these enzymes.

Surprisingly, in our experiments there was no consistent evidence of decreased thyroid function in chicken embryos or chicks that were exposed to PCB 126 throughout embryonic development. All variables measured—UDP-GT activity and plasma thyroid hormones in the present study, as well as thyroid gland mass, gland hormone content and brain for 5'-deiodination activity (McNabb et al. 2001)—supported the conclusion that thyroid development and function were not altered in either embryos or chicks by this exposure to PCB 126. Likewise, PCB 77 exposure during incubation did not affect UDP-GT activity or plasma TH concentrations in chicks at 6 weeks of age.

We know that the PCBs entered the embryos because of other physiological effects seen in dosed birds in our studies. Concurrent studies on other tissues from these same embryos indicated that a dose of 0.13 ng PCB 126/g egg (our second lowest dose) caused immunological alterations and that our highest dose caused 40% mortality (K. A. Grasman, personal communication).

The UDP-GT assay we used was shown to be capable of detecting increases in UDP-GT induced by TCDD in laboratory mice. The pNP substrate is a phenolic
compound that indicates the activity of UDP-GTs toward other phenolic compounds (including both PCBs and T₄). This substrate is conjugated by many UDP-GT isozymes and so is considered to indicate overall induction of UDP-GT-related conjugation. The common use of the assay (which detects multiple UDP-GT isozymes) has led to its use as a frame of reference for Phase II biometabolic activity. However, because this assay is not specific to T₄ glucuronidation, it may not detect subtle effects on the specific isozymes of UDP-GT that are important in thyroid function. The relationship between this measure of activity and the effects on T₄ metabolism remain to be investigated.

Although our studies indicate that exposure to PCB 126 throughout embryonic development does not induce UDP-GT activity in either chicken embryos or chicks, PCB 126 is only one of many different PCB congeners found in polluted environments. Most of the very limited number of studies that have shown alterations in thyroid function in birds have utilized commercial PCB mixtures such as Aroclor 1254, although some studies (Murk et al. 1994b; Gould et al. 1999) have shown decreases in avian thyroid function with exposure to PCB 77. It is important to note that in one of these studies (Gould et al. 1999), PCB 77 and Aroclor 1242 were correlated with negative effects on growth in embryonic chickens, but that the Aroclor 1242 and 1254 treatments were the only ones that resulted in decreased plasma T₄ concentrations. The apparent effectiveness of PCB mixtures in altering avian thyroid function suggests there may be synergistic reactions between the constituents of these mixtures.

**Future Studies**

Because effects of individual PCB constituents and PCB mixtures are not equivalent, it is important that mixtures be used in future studies to examine possible thyroid hormone alterations in birds. It also is important to examine the effects of other PCB congeners, such as the non-coplanar PCB 153, that compose a large percentage of industrial mixtures. Further, to truly determine whether ontogenic alterations occur, it may be necessary to examine more stages throughout embryonic development. Finally, these results are applicable only to the species described—to assess effects, whether in terms of toxicity or endocrine disruption, on birds in general, it will be necessary to examine the same parameters in other avian species.
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Figure 1. Numbering of Possible Chlorine Substitution Positions in PCBs. The diagram shows their relationship to PCB naming protocol.

Figure 2. Chlorine Substitution Positions in PCBs.
Table 1. Naming System for Polychlorinated Biphenyl (PCB) Congeners.

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Figure 3. Diagrams of the Four Coplanar PCB Congeners: PCBs 77, 81, 126 and 169.
Figure 4. The Structure of 2,3,7,8-tetrachlorodibenzo-\(p\)-dioxin (TCDD).

Figure 5. The Structure of Thyroxine (\(T_4\)).
Figure 6. The Hypothalamic-Pituitary-Thyroid (HPT) Axis.
Figure 7. Elution Pattern of Radiolabeled T₄ Metabolites During Sephadex LH-20 Separation Chromatography. The fraction numbers correspond to the following solvents: 1—load (sample in 0.1 N HCl), 2-7—0.1 N HCl, 8-14—H₂O, 15-18—0.1 N NaOH/EtOH (1:1, v/v). Total radioactivity is 18.87% for acid fractions (unbound $^{125}$I), 1.44% for water fractions ($^{125}$I-T₄-G) and 79.70% for alkaline ethanol fractions ($^{125}$I-T₄). Fraction #16 is truncated and accounts for approximately 75.6% of the overall radioactivity recovered.
Figure 8. Standard Curve for Determination of Molar Absorption Coefficient for pNP. The molar absorption coefficient is determined by the slope of the line (14,587/M*cm).
Figure 9. UDP-GT Assay Validation: Proportionality of Enzyme Activity with Enzyme Concentration (Protein Concentration in Homogenate Dilutions) in Adult Quail Liver Homogenate. Data points are the average of triplicates. Error bars show ± 1 standard error. Incubation time was 60 min.
Figure 10. UDP-GT Assay Validation: Time Course for Adult Quail Liver Homogenate $p$NP Assay Validation. Data points are the average of triplicates. Enzyme activity is in µmol $p$NP conjugated/mg protein. Error bars show ± 1 standard error.

\[ y = 0.5305x \]
\[ R^2 = 0.9784 \]
Figure 11. UDP-GT Assay Validation: Time Course for Juvenile Chicken Liver Homogenate pNP Assay Validation. Data points are the average of triplicates. Enzyme activity is in µmol pNP conjugated/mg protein. Error bars indicate ± 1 standard error.

\[ y = 0.7101x \]

\[ R^2 = 0.9339 \]
Figure 12. Induction of UDP-GT Activity toward pNP in TCDD-Treated Mice. Each data point is the average of multiple individuals (N = 6 for control and N = 8 for TCDD-treated). Mice were dosed with either corn oil vehicle or 50 µg/kg body weight TCDD in corn oil intraperitoneally and were sacrificed after 5 days. Enzyme activity is in pmol pNP conjugated/mg tissue/min. Error bars indicate ± 1 standard error. The groups are significantly different from one another (two-tailed t-test for equality of means, equal variances not assumed, p < 0.001).
Figure 13. Profile of Ontogenic UDP-GT Activity in Japanese Quail. The graph shows ages of embryos from day 12 of incubation (-4 on graph) to approximately 42 days posthatch and includes activities during the perihatch period. For the perihatch period, NI samples are indicated at -0.67, I at -0.33 and S at 0 (NI = beak not into air cell, I = beak into air cell, not through shell, S = beak pipped through shell). All data points indicate the mean of individuals (N = 5-17/stage), and error bars indicate ± 1 standard error. Enzyme activity is in pmol pNP conjugated/mg protein*min. * Indicates significant differences from prehatch groups (ANOVA, Tukey’s HSD test, p < 0.001).
Figure 14. Hepatic UDP-GT Activity in Juvenile Chickens Exposed to PCB 126 \textit{in ovo} (combined data). All data points are the average of triplicate samples. The upper graph shows non-injected controls as 0 dose; the lower graph shows injected controls as 0 dose. Enzyme activity is in fmol \textit{p}NP conjugated/mg protein*min.
Figure 15. Hepatic UDP-GT Activity in Juvenile Chickens Exposed to PCB 126 *in ovo* (8/98). All data points are the average of triplicate samples. The upper graph shows non-injected controls as 0 dose; the lower graph shows injected controls as 0 dose. Enzyme activity is in fmol pNP conjugated/mg protein*min.
Figure 16. Hepatic UDP-GT Activity in Juvenile Chickens Exposed to PCB 126 in ovo (10/98). All data points are the average of triplicate samples. The upper graph shows non-injected controls as 0 dose; the lower graph shows injected controls as 0 dose. Enzyme activity is in fmol pNP conjugated/mg protein*min.
Figure 17. Hepatic UDP-GT Activity in Juvenile Chickens Exposed to PCB 126 *in ovo* (1/99). All data points are the average of triplicate samples. This graph shows injected controls as 0 dose; non-injected controls were not used in this experiment. Enzyme activity is in fmol $p$NP conjugated/mg protein*min.
Figure 18. Plasma Thyroid Hormone Concentrations in Embryonic Chickens Exposed to PCB 126. The graphs show combined data for samples from 8/98, 10/98 and 1/99 experiments. Error bars indicate ±1 standard error. *Indicates significant differences from both control values at $p < 0.05$. Injected controls are graphed at dose 0, and non-injected controls are graphed as NI.
Figure 19. Hepatic UDP-GT Activity at Different Doses in PCB 126-Dosed Chickens. Eggs were dosed prior to incubation, and juvenile chickens were sacrificed 42 days posthatch. All data points are the average of triplicate samples. Enzyme activity is in pmol $p$NP conjugated/mg protein*min.
Figure 20. Hepatic UDP-GT Activity at Different Doses in PCB 126- or PCB 77-Dosed Chickens. Eggs were dosed prior to incubation, and juvenile chickens were sacrificed 42 days posthatch. PCB 126-dosed animals were dosed with 0.25 ng PCB/g egg, and PCB 77-dosed animals were dosed with 0.64 ng PCB/g egg. Data bars indicate ±1 standard error. Enzyme activity is in pmol pNP conjugated/mg protein*min. There were no statistically significant differences between controls and either of the treated groups.
Figure 21. UDP-GT Glucuronidation of Labeled T\textsubscript{4} in Control and TCDD-Dosed C57Bl6++ Mice. Mice were either dosed with corn oil as a control or with 50 µg/kg TCDD. Each data point indicates the average of triplicates. Enzyme activity is in pmol T\textsubscript{4} conjugated/mg tissue. Error bars indicate ± 1 standard error.
Figure 22. Validation of Labeled T₄ Assay for Measuring UDP-GT: Time Course Using Adult Quail Liver Homogenate. Each data point indicates the average of triplicates (except for 45 minute sample—duplicates). Enzyme activity is in nmol T₄ conjugated/mg tissue. Error bars indicate ± 1 standard error.
Figure 23. Comparison of Plasma T₄ and T₃ Concentrations with UDP-GT Activity throughout Embryonic Development in Japanese Quail. Perihatch period times are plotted as follows: NI = -0.67, I = -0.33 and S = 0. Enzyme activity is in pmol pNP conjugated/mg protein*min. Error bars indicate ± 1 standard error.
Curriculum Vitae

Ryan J. R. McCleary

Education

April 1993
B.S., Biology, Western Michigan University, Kalamazoo, MI
Minor in Chemistry
Overall GPA: 3.63  Major GPA: 3.73
Undergraduate Honors Thesis: The Use of Aquatic Insect Succession in Determining Post Mortem Interval for Forensic Purposes

December 2001
M.S., Biology, Virginia Polytechnic Institute and State University, Blacksburg, VA
Overall Graduate QCA: 3.71

January 2001-- Ph.D. Program, Zoology, University of Florida, Gainesville, FL
Present
Current GPA: 4.00

Employment

May 1991- August 1991
Summer Intern, Department of Vertebrate Zoology, National Museum of Natural History (Smithsonian), Washington, DC

September 1992- January 1993
Research Assistant, Ecology Laboratory, Western Michigan University, Kalamazoo, MI

May 1993- August 1993
Ed Almandarz Fellow in Herpetology, Lincoln Park Zoo, Chicago, IL

August 1993- July 1994
Graduate Teaching Assistant, General Biology Laboratories, Virginia Tech, Blacksburg, VA
Teaching Evaluations: 3.3, 3.2, 3.6, 3.5, 3.4, 3.7, 3.7/4.0

August 1994- November 1994
Senior Animal Laboratory Technician, International Research and Development Corporation, Mattawan, MI

November 1994- September 1995
Reptile Specialist, Discount Pet Center, Portage and Kalamazoo, MI

June 1995- August 1995
Part-time Instructional Faculty, Kalamazoo Valley Community College, Texas Township, MI
February 1996- February 1997: Funding Specialist, American Auto Funding Corporation, Park Ridge, IL

February 1997- October 1998: Credit Manager and Funding Specialist, General Electric Capital Customized Auto Credit Services, Barrington, IL

January 1999- May 1999: Graduate Teaching Assistant, General and Principles of Biology Laboratories, Virginia Tech, Blacksburg, VA
Teaching Evaluations: 3.3, 3.6, 3.5/4.0

August 1999- December 1999: Graduate Teaching Assistant, Majors Biology Laboratories, Virginia Tech, Blacksburg, VA
Teaching Evaluations: 4.0, 4.0/4.0

January 2000- December 2000: Graduate Research Assistant, Endocrinology Laboratory, Virginia Tech, Blacksburg, VA

January 2001- Present: Graduate Teaching Assistant, Biology Laboratories, University of Florida, Gainesville, FL
Teaching Evaluations: 4.60, 4.86, 4.92, 4.36/5.0

Awards

September 1989- April 1993: Medallion Scholarship, Western Michigan University, Kalamazoo, MI

December 1992: Undergraduate Creative Activities Award, Western Michigan University, Kalamazoo, MI

Memberships in Scientific Societies

January 2001- Present: Zoology Graduate Student Association (ZGSA), University of Florida, Gainesville, FL

January 1999- December 2000: Biology Graduate Student Association (BGSA), Virginia Tech, Blacksburg, VA

November 1999- Present: The Society for Integrative and Comparative Biology (SICB)

April 2000- Present: Sigma Xi, the Scientific Research Society

April 2001- Present: Society for the Study of Amphibians and Reptiles
May 2001-Present  Society for Environmental Toxicology and Conservation (SETAC)

May 2001-Present  American Society of Ichthyologists and Herpetologists

Grants

June 2000  Sigma Xi Grant-in-Aid of Research: Effects of UDP-GT Induction by Exogenous Pollutants on Thyroid Hormone Status in Lab-reared Chickens and Wild Herring Gulls, $800.00. Biology Department matching funds, $500.00


Publications


Published Abstracts


Presentations; No Published Abstract