COMPARISON BETWEEN PCB EXPOSURE AND HYPOTHYROIDISM:
BEHAVIORAL DEVELOPMENT IN SPRAGUE-DAWLEY RATS

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ABSTRACT

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Manufactured to take advantage of their chemical and thermal stability, polychlorinated biphenyls (PCB) are persistent environmental endocrine disruptors used from 1929 to 1977 in the synthesis of plastics, and for coolants in transformers and other dielectrics. Improper handling and disposal has led to environmental contamination and has been linked to physiological, behavioral, and neural disruptions in children and various model species. Many deficits observed in perinatal PCB exposed animals, such as poor memory and subnormal attention proficiency, hypo- and hyperactivity, and poor motor reflexes, are seemingly analogous to other chronic conditions such as congenital hypothyroidism. Using two varying doses of thiouracil to induce hypothyroidism, the present study aimed to compare PCB exposure to hypothyroidism by observing three different behavioral tests. Ultrasonic vocalizations emitted when isolated from the dam, conditioned odor preference (COP) paradigm, and observing an innately sequenced behavior like the grooming chain provide three ways to observe different aspects of social and motor function during development. There was no observed statistical significance in the number of isolation distress calls emitted regardless of treatment. The COP yielded differences between the PCB- and thiouracil-fed animals while the PCB was nearly identical to the control group. Grooming yielded a delay in the formation of complete perfect grooming chains at postnatal day (PND) 15 to 16 yet the PCB and higher thiouracil dose were able to draw nearer to the controls in grooming success by PND 30-31 and 60-61. The current study shows that PCB may be similar to either dose of thiouracil depending on the age of the rat.
I love science, and it pains me to think that so many are terrified of the subject or feel that choosing science means you cannot also choose compassion, or the arts, or be awed by nature. Science is not meant to cure us of mystery, but to invent and reinvigorate it.

-Robert Sapolsky
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CHAPTER I: INTRODUCTION

ENVIRONMENTAL TOXINS

Environmental toxic pollutants are chemical agents suspected to cause birth defects, disease, or death for organisms with which they come into contact in the environment, including humans and other animals (EPA, 2008). These agents usually enter the environment from industries with improper handling or disposal, and in most cases, the chemicals were not known to be harmful until health issues arose in later years. There has been an increase in the correlation between neurological disorders and the prevalence of synthetic chemicals since about 1970 (Colborn, 2004). Bisphenol A (BPA), DDT, and polychlorinated biphenyls (PCB) are examples that have all been in the news recently because of recent studies implicating them in major health concerns such as cancer and developmental problems in children (Newbold et al., 2007; Rothman et al., 1997; Schantz, 1996). The present study aims to focus on the effects of PCB contamination on the behavioral and social development in rat pups after perinatal exposure.

POLYCHLORINATED BIPHENYLS

Polychlorinated biphenyls (PCB) are persistent environmental toxicants that continue to cause neural, physiological, and behavioral problems for many species 30 years after their production was banned in the United States. Beginning in 1929, PCB became widely used in transformers and capacitors as well as plastics, as a result of their nonflammable and insulating qualities (ASTDR, 2000). Though production has ceased, contamination from 209 congeners remains prevalent because of the stable chemical structure (Figure 1) and highly lipophilic properties of PCB (Schantz, 1996). Initial
production and usage, leakage from transformers and capacitors, and improper disposal has led to soil, water, and air pollution producing an environmental toxin capable of causing lasting developmental health problems in many species, including humans (ASTDR, 2000; Khan et al., 2002). PCB accumulates in the adipose tissue of organisms after direct consumption of contaminated food sources. It can then be passed to offspring in-utero prenatally through the placenta or postnatally through lactation (ATSDR, 2000). PCB 47 (2,2’,4,4’-tetrachlorobiphenyl) and PCB 77 (3,3’,4,4’-tetrachlorobiphenyl) were used in the present experiment to represent two common congeners still prevalent in the environment today and two that are tetrahalogenated as is the most abundant thyroid hormone, thyroxine (T4).

![Figure 1: Molecular Structure of Polychlorinated Biphenyl.](University of Illinois at Chicago, 2008)

One hypothesis for the PCB-induced delays in neural development is the effect of this material on thyroid hormone synthesis and metabolism resulting in a depressed thyroid state, or hypothyroidism. During development of a fetus, the thyroid hormones passed from the mother across the placenta are crucial for normal brain development, and minor modifications in amount may induce a dramatic change in a child’s intelligence
Previous studies in this lab have found that PCB causes a dose-dependent depression of thyroid hormone status in rat pups after perinatal exposure (Krishnan, 2007; Donahue et al., 2004; Provost et al., 1999; Juárez de Ku et al., 1994).

**PCB Spills and Contamination**

Several major contaminations have brought PCB exposure to the public’s attention including two mass PCB-poisonings in Yusho, Japan (in 1968) and Yucheng, Taiwan (in 1979) that left around three thousand people ill after ingesting contaminated rice oil. Many people complained of neurological disorders, headaches, and memory loss in addition to the physical deformities that began to appear (Schantz, 1996). Furthermore, growth impairment and developmental deficits were observed in children with prenatal exposure to the contaminated rice oil, including subnormal body weight, slowness, lack of endurance, and subnormal IQ (Winneke, 2002; Schantz, 1996).

The Great Lakes also present a concern for PCB exposure because the toxicant bioaccumulates at the top of the food chain in aquatic animals. This poses a risk for those consuming sport fish including trout and walleye, especially for pregnant women and their children (Stewart et al., 1999). After consumption, PCB accumulates in maternal serum and breast milk and although low levels of PCB were found in pregnant women who did not consume fish, those with fish regularly in their diet had a higher body burden of PCB than those who did not eat fish (Schwartz et al., 1983). Jacobson and Jacobson (1990) continued observations of the subsequent development of children until they reached 11 years of age (Winneke, 2002). They noted the performance of these children on 15 different neurophysiological tests that studied four different aspects of attention and information processing efficiency using short-term memory scanning and mental
rotation. They found that children who were prenatally exposed showed greater impulsivity, poor working memory, and subnormal ability to concentrate on the task at hand (Jacobson and Jacobson, 2003).

**THE THYROID GLAND AND HORMONES**

The thyroid gland produces hormones essential for regulating metabolism, and growth and development (Hadley and Levine, 2007). Maternal thyroid hormones are critical during pregnancy and essential for fetal brain development, particularly before the thyroid gland develops in the fetus and is capable of producing its own thyroid hormones. Hypothyroxinemia and congenital hypothyroidism lead to reduced intelligence, poor memory and attention skills, and reduced motor abilities.

Thyroid hormones are produced and released when the hypothalamus is stimulated to release thyrotropin releasing hormone (TRH) which causes the pituitary to release thyroid stimulating hormone (TSH). TSH binds to follicular cells in the thyroid gland stimulating iodide uptake into thyroid cells. Thyroid peroxidase oxidizes iodide to iodine which is then incorporated into thyroglobulin to form thyroxine (T₄) and triiodothyronine (T₃), the biologically active molecule (Figure 2) (Hadley and Levine, 2007; Zoeller et al., 2007). Deiodinase causes the monodeiodination of T₄, converting it to T₃ in the brain and liver (Morse et al., 1993). The hormones then exert a negative feedback effect on the hypothalamus and pituitary preventing thyroid oversecretion (Hadley and Levine, 2007; Zoeller et al., 2007).
PCB AND ITS EFFECT ON THYROID STATUS

PCB acts as an endocrine disruptor by competing for sites on thyroid transport proteins, thereby displacing T₄ from the protein (Seegal, 2005), and altering the thyroid gland by reducing its ability to respond to TSH (Zoeller, 2001). Coplanar PCB congeners increase T₄ biliary excretion by displacing the T₄ molecules from their binding sites on transthyretin (TTR), a transport protein for thyroid hormones (Khan, 2002; Zoeller, 2001). Fetal PCB exposure is also linked to a decrease in plasma T₄ and an increase in deiodinase activity, thereby causing more efficient conversion of T₄ to T₃. This response is most likely to compensate for the hypothyroid state although it will not protect the brain from damage sustained by hypothyroidism (Morse et al., 1993). Possible causes for some of these disruptions could be a result of the similar structures of PCB and the thyroid hormones, both of which contain two biphenyl rings with substituted halogen atoms (Figures 1, 2).

PCB AND SOCIAL/BEHAVIORAL IMPAIRMENT

PCB exposure has been shown to alter both social and behavioral development dependent upon the dose. Holene et al., (1995) noticed a PCB-induced difference in visual discrimination and hyperactivity after conditioning rats to press a lever following
the onset of a cue light to release a drop of water. PCB 77 has been shown to decrease a female rat’s preference for a sexually receptive male over a female after PCB exposure postnatally via lactation (Cummings et al., 2008). Recent work in our lab demonstrated that a 25 mg/kg dose of PCB induced a significant delay in the formation of complete grooming chains as well as compromising the integrity of the grooming chain (Krishnan, 2007).

Dams exposed to PCB showed an increase in nursing bouts but failed to show high-crouch nursing behavior, a position necessary to allow milk letdown (Cummings et al. 2005). PCB-treated dams also showed an increase in licking and grooming, possibly in an effort to overcompensate for PCB-induced toxic effects (Simmons et al., 2005). A recent study in this lab demonstrated that a 12.5 mg/kg dose of PCB led to a decreased preference of a rat pup for a maternally associated odor (Cromwell et al. 2007).

**PCB AND MOTOR IMPAIRMENT**

Other behavioral abnormalities associated with PCB exposure include deficits in motor coordination as well as behavioral and physiological development (Zoeller, 2001). Krishnan (2007) also showed a motor activity deficit such as loss of strength and coordination while performing a general battery of motor tests. Several studies have implicated PCB exposure with motor ability deficits as well as delays in the righting reflex and negative geotaxis along with decreased grip strength in the hang test (Bowers et al., 2004). There was a reported decrease in motor activity using a figure-eight maze (Goldey and Crofton, 1998).

Hany et al. (1999) saw no changes in the number of lines crossed during their open field tests in juvenile and adult rats exposed to PCB 47 and 77 as well as a mixture
of the two. They noticed that PCB 47 seemed to counteract any changes observed in activity seen in the PCB 77 rats. Another study also found no significant difference in the open field test after exposure to Aroclor1254 using Long-Evans rats (Bushnell et al., 2002). However, Roegge et al. (2004) conducted a battery of motor tests that observed cerebellar-mediated motor function. Using a rotating rod to measure coordination and balance, PCB exposed rats showed a slight deficit indicating that additional areas of motor function should be assessed.

**THIOURACIL**

Anti-thyroid drugs have been around for over 65 years including thiouracil, a chemical known to reduce circulating thyroid hormone concentrations in both clinical (Beck-Peccoz, 2008) and experimental settings (Meserve and Juárez de Ku, 1993). Discovered as an alternative to thyroid gland removal or destruction, thiouracil reduces thyroid hormone by inhibiting the oxidation of iodide to iodine (Beck-Peccoz, 2008; Hadley and Levine, 2007). Thiouracil administered experimentally to cause hypothyroidism results in significantly depressed body weight and adrenal gland mass along with a significantly larger thyroid gland (Meserve and Juárez de Ku, 1993).

**ULTRASONIC VOCALIZATIONS**

By studying ultrasonic vocalizations (USVs), the pup’s negative affective state can be observed for possible signs of increased or decreased anxiety when separated from its mother and allowed to produce isolation distress calls (Wöhr and Schwarting 2008). These calls are emitted by the pup to seek out maternal care such as nursing, licking and grooming, or pup retrieval (Hofer 1996). The emission of 40-kHz USV calls may indicate a more fearful or anxious behavior in adult rats (Knutson et al., 2002) and has
been used as a selective breeding tool for studying the symptoms of some anxiety disorders (Hofer et al., 2001).

**CONDITIONED ODOR PREFERENCE**

Conditioned odor preference (COP) allows observation of the pup’s associative behavior toward a maternal-condition odor and examines the pup’s motivation to seek out its mother (Nelson and Panksepp 1998). Associative behavior is defined as the rat pup’s preference for an individual with an associated cue (Kojima & Alberts, 2009). Rat pups must learn and utilize odor cues before their eyes are open to locate the dam and initiate suckling; therefore they are capable of being conditioned with certain odors simply by pairing it with maternal care which serves as the unconditioned stimulus (Kojima & Alberts, 2009; Nelson & Panksepp, 1996).

**GROOMING SEQUENCE**

Highly sequenced or “syntactic” behavior is common among many mammals, often regulating the simplest behaviors. For instance, rats complete a grooming chain on a routine basis while maintaining structural integrity of this innate sequenced behavior at a frequency 13,000 times greater than chance (Matell et al., 2006). This sequence accumulates over the first two weeks of life with the grooming location developing along the anterior-posterior axis making it ideal for studying developmental disruptions (Berridge et al., 2005).

**GOALS**

The main objective of the present study is to determine if Sprague-Dawley rats exposed pre- and postnatally to PCB demonstrate behaviors statistically similar to or different from those of rats fed a control diet or a diet containing varying doses of
thiouracil. The methods used in this study aim to determine whether PCB or thiouracil-induced hypothyroidism alters different social behaviors or motor ability. Ultrasonic vocalizations and conditioned odor preference are social behaviors developed in response to the pup’s desire to seek out its mother or conspecifics. The fixed action sequence of the grooming chain represents a motor skill that develops within the first 15 days after birth.

**SUMMARY**

Many studies have suggested different ways in which PCB exposure interferes with thyroid status but there are few solid answers (Morse et al., 1993). They still do not explain the physiological deficits experienced by children exposed perinatally to PCB although congenital hypothyroidism has been considered a contributing factor. Few have compared the effects of clinical anti-thyroid drug-induced hypothyroidism to the effects of PCB (Lilienthal et al., 1997). The present study aims to compare social and motor behavior in the rodent model with a dose of PCB known to cause deficits (Krishnan, 2007) and that resulting from two doses of thiouracil in order to gain more insight into the mechanistic action of PCB. By ceasing intake of PCB or thiouracil at weaning and observing three different developmental times, the study can examine the ability of the rats to recover from any developmental deficiencies as a result of perinatal exposure to the thiouracil or PCB.
CHAPTER II: METHODS

ANIMALS

All procedures were approved by the Institutional Animal Care and Use Committee at Bowling Green State University (IACUC protocol #07-013). Sprague-Dawley rats (Harlan Sprague Dawley, Indianapolis, IN) were used for breeding. They were transported to Bowling Green State University at breeding age (approximately postnatal day 80, about 200 g). Females were housed in pairs in clear plastic cages (65 X 24 X 15 cm) until breeding after which they were isolate housed. The animal room was on a 12:12 day/night cycle (lights on at 7:00AM). The room temperature was kept at 20°C with the humidity between 45 and 55%.

After a short period of acclimation to the animal facility, females were mated with males of the same strain. On the first day of pregnancy, as determined by a sperm positive vaginal smear, females were singly housed and placed on a color-coded diet to ensure investigation blindness to pup treatment during testing. The diet consisted of either a control meal (Harlan Teklad Rat Chow #8604, Indianapolis, IN) with no additives, a mixture of equal parts of the tetrachlorinated congeners PCB 47 and PCB 77 (Accustandard, New Haven, CT) to a total concentration of 25 mg/kg, or thiouracil to a concentration of either 1250 mg/kg or 625 mg/kg. Stock PCB was dissolved in absolute ethanol, mixed with 100 g of rat chow, and the ethanol was allowed to evaporate. Equal parts of the stock PCB 47 and PCB 77 were added to the control feed to result in 25 mg/kg of diet provided to the dams. Thiouracil diets were similarly prepared by mixing either 625 mg or 1250 mg 2-thiouracil (Sigma Chemical Company, St Louis, MO) with 1 kg control diet. Litters were standardized to a maximum of ten pups with no fewer than
six pups per litter to avoid major changes in litter dynamics and competition for food among pups. Pups were tattooed in the foot pad at post natal day (PND) 9 for identification and weaning was delayed to PND 25 to ensure pup survival. All animals were provided with control diet beginning at PND 21.

**Behavioral Analysis**

*Isolation Distress Vocalization*

Isolation distress calls are vocalizations made by a rat pup when isolated from the litter and mother, possibly as a sign of anxiety. The rats were separated from the mother and taken into an adjacent testing room. The testing room was kept in darkness except for a red light undetectable by the rats. Each rat pup was placed in a 500 mL beaker under an ultrasonic bat detector (Petterssen D980 ultrasonic detector Uppsala, Sweden) for a one minute habituation on PND 9 and a two minute testing period on PND 10. The ultrasonic vocalization recordings were analyzed offline using a Sonogram (Avisoft Bioacoustics, Berlin, Germany).

*Conditioned Odor Preference*

Conditioned odor preference (COP) is used to measure the pup’s affinity for its mother. COP habituation and testing were carried out in the same testing room and under the same conditions as those used for the isolation distress calls. Each pup was placed individually in a Plexiglas container (18 X 5 X 7.6 cm) visually divided into three sections with metal bars for the floor of the chamber. The chamber rests upon two glass jars with a cotton ball in each jar. A lamp was added above the chamber for extra light during filming. On PND 12, pups were habituated in the testing chamber for one minute.
Conditioning began on PND 13 with three hours of maternal deprivation followed by a 30 minute conditioning session. The litter was divided into two groups with one group conditioned in a novel cage with four cotton balls, the other group with the mother in an identical but novel cage. In the non-maternal cage, one cotton ball was placed in each corner and moistened with 0.25 mL of pure lemon extract (The Kroger Co., Cincinnati, OH). In the maternal cage, one mL of pure lemon extract was applied to the ventral surface of the mother immediately before she was placed in the novel cage. This conditioning bout was repeated two more times during the days each after another three hour maternal deprivation period. After the third conditioning bout, pups were returned to the home cage. The mother was bathed with generic hand soap to eliminate the lemon scent and returned to the home cage as well.

COP testing was conducted on PND 14 in the same chamber in which the pups were habituated. Testing was conducted after three hours of maternal deprivation. One side of the chamber rests above a jar containing a cotton ball saturated with 1 mL of lemon extract while the other jar contains a cotton ball saturated with 1 mL of distilled water. The location of the two scents was alternated throughout the testing to ensure no place preference. The pup was placed in the center chamber and allowed to move about freely for five minutes while being filmed using a commercially available digital video recorder. Analysis was conducted offline by a trained observer blind to pup treatment, pup conditioning, and scent location.
**Novel Odor**

Novel odor testing was conducted immediately following COP conditioning and tested the pup’s affinity toward the lemon scent or a novel odor, peppermint extract in this case. Two wooden rectangular alleys were visually divided into three unequal sections: start (7 X 7 cm), middle (24 X 7 cm), and end (7 X 7 cm). A strip of Plexiglas was placed over each alley to minimize odor dispersal (Nelson and Panksepp, 1996). Each end section contained a small glass bottle with half of a cotton ball placed just inside the neck of the bottle. The cotton was saturated with either 1 mL of pure lemon extract or 1 mL of peppermint extract. Timing began after a pup was placed in the starting section of chamber, and ended when the nose of the pup crossed into the end section or when 60 seconds passed. Five timed trials were conducted for each scent per pup and the trials were counterbalanced between scents. Data were analyzed offline by a trained observer blind to the pup treatment.

**Behavioral Action Syntax**

**Grooming Sequence**

On PND 15 and 16, the pups were placed individually in a grooming chamber in order to observe grooming chains. A clear Plexiglas cage was placed over a mirrored stand and pups were habituated for the first five minutes. After habituation, two to three drops of water were placed on the dorsal surface of the pup to elicit a grooming response. Pups were filmed then for an additional ten minutes with a commercially available digital video recorder.

For the grooming sessions between PND 30-32 and PND 60-62, pups were filmed for two grooming sessions. The pups were placed in a cylindrical grooming chamber
above the mirrored stand to avoid the rats stationing in the corners. The rats again had five minutes of habituation to the chamber and then were misted on the dorsal surface with a commercial spray bottle to elicit a grooming response. The ten minute grooming session was recorded and DVDs were analyzed offline.

**Grooming Syntax**

A grooming chain has occurred after the correct sequence of four grooming phases has been carried out by the rat pup. Phase I begins with five to nine short, quick bilateral swipes of the front paws around the nose and mouth (referred to as ellipses). Phase II is one to four short asynchronous strokes of the forelimbs along the mystacial vibrissae. Phase III follows with three to ten synchronous strokes extending from behind the ears to swipe over most of the head. Lastly, phase IV begins when the rat turns and commences body licking over the lateral portion of the flanks (Figure 3) (Cromwell and Berridge 1996).

![Figure 3: Phases of Syntactic Grooming Chain](Matell et al., 2006)
Grooming Video Analysis

Each grooming session was analyzed using slow motion (frame by frame) technology to ensure determination of completion of the entire grooming chain. A complete perfect chain was defined as the animal having completed all four phases in correct order, with the first three phases being completed in five seconds or less, without any interruptions. An incomplete chain began with phase I, but may have skipped phases, completed out of order, or simply been revised to a flexible bout. A flexible bout was defined as any time a rat begins grooming whether or not a chain was initiated. Flexible bouts included non-grooming chains such as those that did not begin with phase I. An incomplete chain was also considered for any grooming syntax where the first three phases may take longer than five seconds or the rat stops to paw lick between phases III and IV. An imperfect chain began with phase I and progressed through phase III correctly but the rat did not show typical body licking and instead licked their forelimbs, ventral surface, or genital region. Total grooming time included syntactic chains and all the flexible bouts observed during scoring (Cromwell and Berridge 1996).

EUTHANASIA

At approximately PND 65, rats were anesthetized with Fatal-Plus™ (Vortech Pharmaceuticals, Dearborn, MI) and then terminated by decapitation to collect trunk blood and vital organ weights. Immediately after decapitation, the blood was collected in conical tubes and centrifuged for 30 minutes at room temperature to collect blood serum for hormone analysis. Serum samples were frozen at -20°C until thyroid hormone analysis could be performed. Final body weights as well as the weight of the pituitary, thyroids, adrenals, and liver were recorded.
**THYROID HORMONE ANALYSIS**

Hormone assays were conducted for the two thyroid hormones: triiodothyronine ($T_3$) and thyroxine ($T_4$). The assays were completed using kits and instructions from MP Biomedicals (Orangeburg, NY) and a microtiter plate reader (Victor$^2$ 1420 Multilabel Counter, Wallac Laboratories, Turku, Finland) was used to read the absorbencies of the assays at 450 nm as per the instructions.

**STATISTICAL ANALYSIS**

All statistical analysis was performed using SPSS software program (Chicago, IL). Analysis of variance (ANOVA) was performed on individual pup means to determine the presence of significant differences ($p<0.05$). After determining significant main effects and interactions, a Tukey post hoc test was run for pairwise comparison of means. A repeated measures general linear model was used to determine significant differences for COP, NO, and grooming within each diet and between diets as well. Pairwise t-tests and independent sample t-tests were used to determine appropriate significance.
CHAPTER III: RESULTS

ULTRASONIC VOCALIZATIONS

An analysis of variance (ANOVA) revealed no significant differences in number of isolation distress calls (Figure 4). Litter means varied widely within diets especially the control (litter means for controls = 110.9, 200.3, 193.7, 69.6, 61.3, 43.6, 38.3, 255.3, 138.0, and 196.6 calls/120 seconds) indicating that there is something besides the diet affecting their calls. Despite the variation, there was a trend for subnormal number of calls per 120 seconds by the low dose thiouracil and the PCB animals.

Figure 4: Mean number of individual ultrasonic vocalizations: Isolation distress calls emitted by 10 day old rats during a 120 second period. Control: N = 81 (10 litters), 625 mg/kg thiouracil: N = 39 (5 litters), 1250 mg/kg thiouracil: N = 47 (7 litters), 25 mg/kg PCB: N = 48 (7 litters). Error bars represent standard error of the mean.
CONDITIONED ODOR PREFERENCE

A general linear model of repeated measures revealed a significant main effect of the number of entries (F(1,189) = 5.234 p<0.05) and a significant main interaction between entries, treatment, and sex on the number of entries into the compartment containing either odor (F(1,189) = 3.007 p<0.05). Paired sample t-tests revealed that the PCB exposed males conditioned with the cotton had a significant increase in the number of entries into non-scented side of the testing chamber as to the lemon-scented side (t(12) = 2.842 p<0.05). There was no observed significant main effect between diets (Figure 5).

After it was determined with a repeated measures test that there was no significant gender difference, all compartment duration data combined results from both males and females. A significant main effect within diets for duration was detected (F(1,189) = 12.893 p<0.005) as was a significant main interaction between duration and conditioning (F(1,189) = 15.885 p<0.005). Paired sample t-tests revealed a significant increase in the time spent on the non-scented side of the testing chamber for controls (t(39) = 2.607 p<0.05), 625 mg/kg thiouracil (t(20) = 3.302 p<0.005), and 25 mg/kg PCB (t(23) = 3.214 p<0.005).
Figure 5: COP - Mean number of entries into each compartment based upon associative history. Control: N = 80 (10 litters), 625 mg/kg thiouracil: N = 43 (6 litters), 1250 mg/kg thiouracil: N = 33 (5 litters), 25 mg/kg PCB: N = 49 (7 litters). *Denotes significance p<0.05 between cotton-conditioned rats and entries into lemon or nonscented side. Error bars represent standard error of the mean.
Figure 6: COP - Mean Duration spent in each compartment dependent on associative history and sex. Control: N = 80 (10 litters), 625 mg/kg thiouracil: N = 43 (6 litters), 1250 mg/kg thiouracil: N = 33 (5 litters), 25 mg/kg PCB: N = 49 (7 litters).

* Denotes significance within diets from duration spent in lemon side of compartment: 
  * = p<0.05, *** = p<0.005. Error bars represent standard error of the mean.
NOVEL ODOR

The novel odor approach indicated that all overall, rat pups approached the lemon scent more quickly than they approached the peppermint regardless of whether lemon scent was associated with mother or cotton ball (Figure 7). A significant main effect depending on associative history was detected in both the latency to approach either the lemon (cotton-conditioned: $F(3,84) = 4.016, p<0.05$; mom-conditioned: $F(3,84) = 3.006, p<0.05$) or the novel odor (cotton-conditioned: $F(3,84) = 4.28, p<0.01$). The shortest latency to approach the lemon scent in the dam-conditioned controls was seen compared to the other diets (Figure 8). However, PCB-fed animals were not significantly different from controls in learning to associate lemon with the dam supporting earlier COP data. The 1250 mg/kg thiouracil-fed rats had the greatest latency to approach the lemon after being conditioned with the dam (control: $p<0.005$; 625 mg/kg thiouracil: $p<0.05$; and 25 mg/kg PCB: $p<0.05$).
Figure 7: NO - Mean latency to approach a conditioned or novel scent: Not dependent on associative history. Control: N = 36 (10 litters), 625 mg/kg thiouracil: N = 18 (5 litters), 1250 mg/kg thiouracil: N = 15 (5 litters), 25 mg/kg PCB: N = 16 (5 litters). *Denotes significance from controls p<0.05, **denotes significance from controls p<0.01. Error bars represent standard error of the mean.

Figure 8: NO - Mean latency to approach a conditioned or novel odor: Dependent upon associative history. Control: N = 36, 625 mg/kg thiouracil: N = 18, 1250 mg/kg thiouracil: N = 15, 25 mg/kg PCB: N = 16. *Denotes significance from controls p<0.05, **denotes significance from controls p<0.01, +denotes significance from 625 mg/kg thiouracil p<0.05. Error bars represent standard error of the mean.
GROOMING

A general linear model showed no gender effects so all analyses for grooming was carried out with combined data from males and females. A one-way ANOVA revealed no significant main effects of diet on total grooming time (Figure 9) and for mean incomplete grooming chains (Figure 10). While no main significant effect was observed, independent sample t-tests revealed a trend at PND 15-16 for both the 625 mg/kg thiouracil ($t(14) = 2.55$, $p<0.05$) and 25 mg/kg PCB ($t(22) = 3.208$, $p<0.005$) groups.

With regard to the percentage of complete perfect grooming chains, there was a significant main effect at PND 15-16 (Figure 11) ($F(3,43) = 11.887$, $p<0.005$). Complete perfect chains were absent with the 625 mg/kg thiouracil dose ($p<0.005$) and 25 mg/kg dose of PCB ($p<0.005$) while the 1250 mg/kg thiouracil dose had a 1.19% mean completion rate ($p<0.005$). While there were no observed significant main effect between diets in the number of complete imperfect chains (Figure 12) and overall complete chains (Figure 13), independent sample t-tests revealed an observed trend in the decreased production of overall complete chains which included complete perfect and complete imperfect (625 mg/kg thiouracil: $t(14) = 2.55$, $p<0.05$; 1250 mg/kg thiouracil: $t(18) = 3.16$, $p<0.01$; 25 mg/kg PCB: $t(22) = 3.208$, $p<0.005$). While there were no significant treatment effects on grooming chain completion at PND 30-31 or 60-61, there was a trend toward continued depression by thiouracil exposed animals of complete perfect (Figure 11) and overall complete chains (Figure 13). The 625 mg/kg thiouracil rats also showed a significant depression ($F(3,31) = 3.575$, $p<0.05$) in chains initiated per minute compared to the 25 mg/kg PCB rats at PND 30-31 (Figure 14).
Figure 9: Mean total time spent grooming.
Grooming was observed at three different developmental time periods. Control: N = 8 (2 litters), 625 mg/kg thiouracil: N = 8 (2 litters), 1250 mg/kg thiouracil: N = 12 (3 litters), 25 mg/kg PCB: N = 16 (4 litters). * Denotes significance from controls p<0.05. Error bars represent standard error of the mean.
Figure 10: Mean Percentage of Incomplete Grooming Chains.
Recorded for each developmental time period. Control: N = 8 (2 litters), 625 mg/kg thiouracil: N = 8 (2 litters), 1250 mg/kg thiouracil: N = 12 (3 litters), 25 mg/kg PCB: N = 16 (4 litters).
Figure 11: Mean Percentage of Complete Perfect Grooming Chains. Recorded for each developmental time period. Control: N = 8 (2 litters), 625 mg/kg thiouracil: N = 8 (2 litters), 1250 mg/kg thiouracil: N = 12 (3 litters), 25 mg/kg PCB: N = 16 (4 litters). ***Denotes significance from controls p<0.005.
Figure 12: Mean Percentage of Complete Imperfect Grooming Chains. Recorded for each developmental time period. Control: N = 8 (2 litters), 625 mg/kg thiouracil: N = 8 (2 litters), 1250 mg/kg thiouracil: N = 12 (3 litters), 25 mg/kg PCB: N = 16 (4 litters).
Figure 13: Mean percentage of overall complete (perfect + imperfect) grooming chains. Recorded for three developmental time periods. Control: N = 8 (2 litters), 625 mg/kg thiouracil: N = 8 (2 litters), 1250 mg/kg thiouracil: N = 12 (3 litters), 25 mg/kg PCB: N = 16 (4 litters). *Denotes nonsignificant trend from controls: * = p<0.05 for 625 mg/kg thiouracil, ** = p<0.01 for 25 mg/kg PCB, *** = p<0.005 for 1250 mg/kg thiouracil.
Figure 14: The Number of Chains initiated per Minute.
Recorded over three developmental time periods. Control: N = 8 (2 litters), 625 mg/kg thiouracil: N = 8 (2 litters), 1250 mg/kg thiouracil: N = 12 (3 litters), 25 mg/kg PCB: N = 16 (4 litters). *Denotes significance from 625 mg/kg thiouracil p<0.05. Error bars represent standard error of the mean.
THYROID HORMONE ASSAYS

Treatment with either thiouracil or PCB during gestation and lactation resulted in both thyroid hormones ($T_4$ and $T_3$) being elevated in concentrations in adult rat blood serum. For $T_4$ concentrations (Figure 15), a one-way ANOVA determined a significant main effect among diets ($F(3,25) = 6.13, p<0.005$). Animals fed 25 mg/kg PCB resulted in levels that were significantly greater than those in either treatment from control ($p<0.005$) or the 625 mg/kg thiouracil animals ($p<0.05$). Furthermore, a one-way ANOVA also revealed significant main effects for $T_3$ concentrations depending on diet ($F(3,25) = 13.717, p<0.005$) Experimental diet feeding and withdrawal resulted in $T_3$ levels significantly greater than those in controls regardless of treatment (Figure 16). The 25 mg/kg PCB rats had $T_3$ concentrations over twice that of controls while those exposed to thiouracil were less drastically affected (control: $p<0.005$; 625 mg/kg thiouracil: $p<0.005$, 1250 mg/kg thiouracil: $p<0.05$).
Figure 15: Mean Serum T4 concentrations. Obtained PND 62 to 65. Control: N = 6 (3 litters), 625 mg/kg thiouracil: N = 6 (3 litters), 1250 mg/kg thiouracil: N = 6 (3 litters), 25 mg/kg PCB: N = 8 (4 litters). ***Denotes significance from controls p<0.005. Error bars represent standard error of the mean.

Figure 16: Mean Serum T3 Concentrations. Obtained PND 62 to 65. Control: N = 6 (3 litters), 625 mg/kg thiouracil: N = 6 (3 litters), 1250 mg/kg thiouracil: N = 6 (3 litters), 25 mg/kg PCB: N = 8 (4 litters). *Denotes significance from PCB: p<0.05 = *, and p<0.005 = ***. Error bars represent standard error of the mean.
CHAPTER IV: DISCUSSION

ULTRASONIC VOCALIZATIONS

Very few studies have investigated the effects of PCB on early natural social behavior (Cromwell et al., 2007). The present study presented two different social behaviors that did not focus on reward, but rather on the affective state of the pup and its desire to seek out its mother and explore. No obvious alterations were observed in the number of ultrasonic vocalizations emitted by the rat pups while isolated from the dam as a result of thiouracil or PCB treatment (Figure 4). There was great range in the mean number of calls emitted per litter for all diets, particularly within the group receiving the control diet. The order in which the pups were tested did not account for this variability and they were all tested at approximately the same time of day.

Studies of ultrasonic vocalizations including previous work in our lab and elsewhere have not been consistent with regard to the strain of rat used (Long-Evans - Harmon et al., 2008; Wistar - Wöhr and Schwarting, 2008) which could call differently than the Sprague-Dawley rats used in the present study. The acoustics in the testing room along with possible variations in detectable background noise could also play a role in the variability observed. However, it is possible that something else is affecting the animals and subsequent testing should be done for more days.

CONDITIONED ODOR PREFERENCE

As pups develop, they are less likely to call for maternal care but rather seek her or litter mates using exploratory behavior and associated odor cues (Nelson and Panksepp 1996). This behavior is formed from interactions with the dam and conspecifics between PND 1 and 9 so that by about PND 15, rat pups are able to discriminate between huddling with litter mates versus with other animate or inanimate sources of heat (Kojima and
Alberts, 2008). One measure within the COP paradigm is the amount of motor activity inside the testing chamber by observing the number of times the rat pup crosses either line into the lemon-scented or non-scented (water) compartment. Overall, the rat pups in the present study entered each compartment nearly equally regardless of associative history with the exception of the cotton conditioned PCB males (Figure 5). Diet did not appear to hinder motor activity or exploratory behaviors given that the number of entries was relatively consistent across treatments. Anxious rats are less likely to explore their surrounding (Ohl et al., 2001), illustrating that diet in the present study had no significant main effect on anxiety seeing as they were all similar for number of entries.

The length of time spent in each chamber is a good indication of the rat pup’s ability to learn and associate a novel odor with the dam, in this case lemon (Nelson and Panksepp, 1996). Pups continue to explore both sides of the chamber but the control rat pups conditioned to associate lemon with the mother should spend more time within the lemon compartment (Cromwell et al., 2007; Nelson and Panksepp, 1996). Only the PCB-exposed males who had been conditioned with the dam displayed this preference significantly and thiouracil-exposed females conditioned with the dam actually showed a slight aversion to the lemon scent (Figure 6). This shows a difference in the interaction of PCB and thiouracil on observed behavior. The control, 625 mg/kg thiouracil, and PCB-fed animals conditioned with cotton balls all showed a significant preference for the non-scented compartment over the lemon, indicating that they were not conditioned with any residual lemon scent not washed away before the dam was placed back in the home cage with the entire litter.
The PCB-fed males conditioned with the dam showed a significantly greater preference for the lemon scent compared to both control and 1250 mg/kg thiouracil-fed males with the same associative history. Differences observed between the present data and previous lab work (Cromwell et al., 2007) could be a result of low versus high doses of PCB and complex inconsistent interactions between PCB and thyroid status (Bansal and Zoeller, 2008). A future study should examine the USV emission during the COP paradigm to better understand each individual pup-dam relationship.

**NOVEL ODOR**

In order to make sure that the rat pups were being conditioned to the maternal associated odor, after COP pups underwent a novel odor approach (Nelson and Panksepp, 1996). Regardless of associative history, there is evidence that the pups preferred the lemon scent to the novel or peppermint odor (Figure 7). A significant increase in the latency to approach each scent in both thiouracil diets indicates that controls learned the associative odor and therefore support COP findings. The PCB and thiouracil treated rats differed statistically indicating that PCB may be affecting behavioral development differently than hypothyroidism (Figure 8).

**GROOMING**

Between PND 15 and 16, the 625 mg/kg thioruacil and 25 mg/kg PCB animals were unable to complete grooming chains and rather than flank lick, they lost their balance and fell over, licked their belly or forelimbs, or stopped grooming after phases II or III (Figure 11). The 1250 mg/kg dose showed a very small percentage of complete chains (one rat was able to complete one grooming chain) and overall spent less time grooming at PND 15-16 (Figure 9). The PCB-fed rats caught up to the controls and their
results at PND 30-31 and at PND 60-61 indicate a possibility to overcome toxicity effects over time. The 625 mg/kg thiouracil group was unable to complete chains and averaged far fewer chains initiated per minute at the older age groups than any other group and significantly fewer than the PCB group (Figure 14). The 1250 mg/kg thiouracil average of complete perfect chains caught up to the control and PCB groups by PND 60-61 and showed only a slight decrease at the two later developmental periods. With a decreased number of complete perfect chains, the 625 mg/kg increased the amount of incomplete (Figure 10) and complete imperfect chains (Figure 12).

These findings were similar to those in previous work in the lab which found that 25 mg/kg PCB exposed rats showed a significant delay in the correct formation of complete grooming chains. Their 0% completion rate at PND 15-16 improved over the next two developmental time periods with only a slight difference as compared with controls (Krishnan, 2007). Grooming chains constitute an innately sequenced behavior that requires coordination and balance in order to complete the entire chain. This coordination of highly sequenced behavior results from the neurotransmission of dopamine in the basal ganglia (Matell et al., 2006). Studies have shown that PCB can disrupt dopamine synthesis in the basal ganglia by altering tyrosine hydroxylase, an enzyme involved in dopamine synthesis (Lyng et al., 2007).

The loss of balance while grooming may be a result of cerebellar alterations in the developing brain. Thyroid hormones are important in brain development and PCB has the capacity to reduce these circulating levels, thus leading to shifts in neuronal growth (Roegge et al., 2006). Laboratory studies have observed loss of low frequency hearing as a result of outer cell hair loss in the basilar membrane of the cochlea (Crofton et al.,
2000). The evidence of hearing loss may extend to deficits in vestibular function, affecting balance during complex motor functions such as the grooming sequence. Berridge (1989) determined that complete cerebellar extraction did not alter the integrity of the grooming chain but rather that this structure could play a role in the necessary balance and coordination to carry out complex motor action.

**THYROID HORMONES**

Enzyme immunoassay revealed significant alterations in thyroid hormone concentrations resulting from dietary treatments. Increasing amounts were observed in the order: controls < 625 mg/kg thiouracil < 1250 mg/kg thiouracil < 25 mg/kg PCB when measured between PND 62 - 65. Some studies suggest that PCB disrupts thyroid hormone receptor gene expression which could lead to resistance. This results in an increase in free $T_4$ and $T_3$ as a result of decreased target tissue response to the hormones (Kuriyama et al., 2003). However, the general behavioral tests conducted in the present study indicate that this may not be the problem. The large increase in thyroid hormone levels in adults could result from recovery from early toxicity. The thyroid gland and its regulatory mechanisms overcompensate in an effort to overcome toxic effects.

Although elevated by all treatments, serum $T_4$ concentrations were not significantly altered, except in PCB animals which were significantly elevated (Figure 15). Similarly, PCB exposed animals displayed the greatest level of serum $T_3$ concentrations (Figure 16). Smaller, but significant compensation of $T_3$ concentration was exhibited in thiouracil exposed rats. A possible explanation in PCB animals could be a result of PCB increasing the activity of Type II deiodinase resulting in a greater conversion of $T_4$ to $T_3$ (Morse et al., 1993). Thiouracil is known to decrease deiodinase
activity resulting in higher T₄ and lower T₃ concentrations and its withdrawal may result in a rebound in this deiodinase activity producing more T₃ (du Mont et al., 2001).

This recovery effect could explain why the rats exposed to the 1250 mg/kg thiouracil and 25 mg/kg PCB are able to finish nearly the same amount of complete grooming chains by PND 60. The lower 625 mg/kg thiouracil dose did not have as great a recovery effect as the higher dose which could explain why the lower dose was significantly different from both the control and PCB. The lower thiouracil dose also had displayed greater total grooming time, although not significantly altered whereas the other three were similar in time spent grooming.

CLINICAL IMPLICATIONS

As the number of children diagnosed with autism each year increases without explanation, a broad-based research effort is searching for a cause and eventually a way to decrease or eliminate this trend (Woodward, 2001). Many factors including genetics, immunizations, and environmental contaminants have been blamed as the catalyst; however, diagnosis is based on behavioral assessments and no treatment or cure exists (Lainhart et al., 2002; Chez et al., 2004; Woodward, 2001). Rats exposed to PCB exhibit some of the symptoms associated with autism such as hyperactivity, clumsiness, and obsessive-compulsive tendencies. In the present study, PCB-fed rat pups had the greatest number of grooming chains per minute at PND 15-16 and 30-31 although not significantly greater than the other groups. PCB rat pups also showed less coordination and balance in the earliest stages of grooming as well. This clumsy action that did not allow them to successfully complete the grooming chain, yet they would attempt it over and over again. This is evident by the significantly greater proportion of incomplete
chains observed at PND 15-16. However, the PCB group appears to overcome some of the toxic effects by PND 60-61, and shows the fewest chain initiations per minute at that age. Future studies should be completed with the grooming model to rule out coordination and balance as the main reason for the elevated number of incomplete chains. While the rats studied were certainly not autistic, the similarities between some of the observed behaviors and symptoms present in autistic spectrum disorder promote further studies to look more closely at toxic pollutants such as PCB as a possible environmental trigger.

CONCLUSIONS

The present study indicates that PCB interactions on the thyroid gland could result in symptoms similar to those of thiouracil-induced hypothyroidism. Compared to the two doses of thiouracil used in the study, it was observed that PCB may be similar to either the high dose or low dose of thiouracil depending on the age of the rat. Further studies should examine more developmental studies over time and more consistently monitor thyroid hormone concentration throughout development.
REFERENCES


