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THE IMPACT OF POLYCHLORINATED BIPHENYLS ON MATERNAL CARE AND OFFSPRING SEXUAL BEHAVIOR IN LABORATORY RATS – THE IMPORTANCE OF EXAMINING ALL COMPONENTS OF A DYNAMIC SYSTEM

presented by

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has been accepted towards fulfillment
of the requirements for the

Doctoral degree in Zoology

Major Professor's Signature

May 12, 2006

Date

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By

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Zoology

2006

ABSTRACT

THE IMPACT OF POLYCHLORINATED BIPHENYLS ON MATERNAL CARE AND OFFSPRING SEXUAL BEHAVIOR IN LABORATORY RATS – THE IMPORTANCE OF EXAMINING ALL COMPONENTS OF A DYNAMIC SYSTEM

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Polychlorinated biphenyls (PCBs) are persistent environmental contaminants that remain in the environment today even after their production was banned in the United States in the 1970s. While many studies have examined the effects of PCBs on a variety of behaviors and developmental outcomes, the effects of the contaminants on maternal behavior have been largely ignored. Those that have evaluated maternal behavior after gestational exposure to PCB used the traditional paradigm in which a dam is treated while she is pregnant and the resulting maternal behavior is evaluated. With this approach, it is impossible to distinguish between effects on maternal behavior that are the result of direct actions on the dams' endocrine and/or nervous systems from those that stem from indirect effects mediated by changes in the physical and behavioral profiles of the exposed litter. In this dissertation, I examined the effects of different doses of the dioxin-like PCB congener 3, 4, 3', 4'-tetrachlorobiphenyl (PCB 77) on maternal behavior in rats using a cross-fostering paradigm to gain insights about the contributions of direct and indirect effects of PCB 77 exposure on maternal behavior. I also examined the effects of the toxin on offspring partner preference and sexual behavior.

Various components of maternal behavior were affected when dams were treated with a dose of 2 mg/kg b.w. from gestation day (GD) 6 – 18. Some of the effects on maternal

behavior can be ascribed to prenatal exposure of the litters to the PCB, while other effects appear to be due to both direct effects of the PCB on the dams and effects mediated by changes in the offspring.

A second experiment employed a dose of 3 mg/kg to determine how an intermediate dose of PCB 77 affects maternal behavior and to expand the behavioral analysis. The results from this experiment confirm that exposing dams to PCB during gestation alters the care they provide to their litters, and that many of the affected behaviors appear to be the result of dams rearing prenatally PCB-exposed pups instead of being due to the treatment the dams received while pregnant.

Finally, we examined the sexual behavior and partner preference of offspring that were exposed to PCB pre- and/or postnatally. While PCB exposure did not affect male or female sexual behavior, it did alter the display of partner preference behavior. These effects were more salient in the females than the males.

This dissertation also addresses the traditional experimental paradigm that is often used, in which treatment of the dam is simply considered an avenue through which toxins can be delivered to the developing embryo without taking into consideration any possible alterations in the dams' behavior. Since such exposure is not specific to the pups the effects of the contaminant on the dam must be evaluated in terms of its impact on the offspring.

ACKNOWLEDGMENTS

I would like to thank the people who made this undertaking possible, starting with my committee: my advisor, Dr. Lynwood Clemens, for helping to guide me through this incredible journey and for knowing when and how hard to push me; Dr. Antonio Nunez, for the guidance and countless number of manuscript edits; Dr. Cynthia Jordan, for being there to help me through both professional and personal situations; and Dr. Joseph Lonstein, for his incredible knowledge of maternal behavior.

I would like to thank my family: my husband, for having faith in me even when I don't have faith in myself, and without whom I wouldn't have made it through my first graduate course; my parents, for always believing in me and letting me know; and my daughter, who helped me to get through graduate school by reminding me what's really important in life.

I am very thankful for those in my lab who helped me with a variety of tasks: Dr. Yu-Wen Chung-Davidson, who taught me a number of laboratory techniques; Sawyer Pouliot and Nancy Carlisle, for their help with maternal behavior video analysis; and Sarah Paxton, for her assistance with daily laboratory activities. I would like to thank Casey Henley for being my office mate and lab mate for 6 years, and all that that entails, and for teaching me how to properly throw a softball; and Jamie Johansen, for everything.

I would also like to thank everyone in the Zoology Department and Neuroscience Program for their support; the Graduate School for my Food, Nutrition, and Chronic Disease Dissertation Completion Fellowship; and the Biological Sciences Department for employing me for many semesters. My research was supported by a NIEHS Superfund grant (P42ESO4911) and a NSF grant (IBN9728883) to L. Clemens.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Chapter 1. Introduction

PCBs – The Nature of the Problem

Polychlorinated biphenyls do not burn and are nearly indestructible – characteristics that originally made them perfect for use in transformers and insulators, coolants and hydraulic fuels. These same characteristics are why they are considered to be one of the worst environmental villains of the twentieth century. Thirty years after their manufacture was banned, polychlorinated biphenyls (PCBs) continue to move throughout the world on winds, waves and rivers and threaten the destruction of entire populations of humans as well as non-humans. The global pattern of distribution of PCBs is so dynamic and unceasing that regions of the world far from where PCBs were used are suffering the most damage – damage that continues to worsen with every year.

The safe disposal of these chemicals is a persisting challenge. Thousands of old transformers still exist today, harboring more than 100 million pounds of PCBs. All it takes is for a fire to erupt near one of these transformers, and poisonous chemicals spray into the air and leak into the pavement. The molecules seep into our groundwater or catch a ride on the closest gust of wind and are lifted up into the atmosphere where they can travel hundreds of miles. When the air cools, the droplets condense and fall to the ground where they can move easily with the currents. The cycle continues until the contaminants reach a cold place where they can rest in liquid form. As it turns out, this

place happens to be one that is considered to be one of the most untouched, pristine places on Earth – the Arctic.

An estimated 67 tons of PCBs arrive in the Arctic every year where about two-thirds remains. PCBs are lipophilic (i.e. accumulate in adipose tissue) and work their way up the food chain by magnifying in concentration as they get closer to the top. The people of the Arctic are most vulnerable not only because where they reside is a virtual dumping ground for the PCBs, but also because of their place at the top of the world's dietary hierarchy. They sustain themselves on sea otters and whale, animals that are themselves near the top of the food chain and have a much higher concentration of PCBs compared to land-raised animals such as beef and poultry. Because of this, Inuit women carry such large loads of these chemicals that their bodies and breast milk could be classified as hazardous waste, and children of the Arctic suffer extraordinarily high rates of infectious diseases such as ear infections that recur so often that they cause permanent hearing loss (Cone, 2005).

The detrimental effects of PCBs on these people are a clear concern and illustrate that no one is safe from these toxins. According to Sheila Watt-Cloutier, chair of the Inuit Circumpolar Conference, "the Arctic is the barometer of the health of the planet, and if the Arctic is poisoned, so are we all" (Cone, 2005).

PCBs – The Intracellular Mechanism

There are 209 different PCB congeners that vary both in the number and location of chlorine molecules. According to Safe (1990), the most toxic type of PCBs have a coplanar, non-*ortho*-substituted structure, and are similar to 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD; Safe, 1990). While PCB has recently been shown to directly activate estrogen receptor (ER) alpha (Abdelrahim *et al.*, 2006), most of the toxic and biological effects of PCBs are mediated by the aryl hydrocarbon receptor (AhR) (Safe *et al.*, 1998; Mimura and Fujii-Kuriyama, 2003). The AhR is an intracellular cytosolic protein that is ligand-activated and binds with high affinity to TCDD or related compounds (i.e. coplanar PCBs; Safe *et al.*, 1998). After it is activated by the ligand, the AhR dissociates from its chaperone complex and moves into the nucleus, where it binds to the AhR nuclear translocator (ARNT) and then to the DNA recognition site where it can regulate the transcription of a number of genes. Currently, there are no known endogenous ligands for the AhR (Pravettoni *et al.*, 2005).

TCDD and structurally-related compounds are considered to be endocrine disruptors. They have the ability to modulate several endocrine response pathways by inducing AhR gene expression and inhibiting estrogenic functions and ER-mediated signaling (Safe *et al.*, 1998). For example, AhR-induced gene expression has been shown to decrease ER levels (Mortensen *et al.*, 2006), inhibit cell proliferation and gene expression in breast, endometrial and ovarian cancer cells (Safe *et al.*, 2003), and induce germ cell apoptosis and diminish ovarian follicular reserves (Franczak *et al.*, 2006). The mechanisms by

which the AhR mediates changes in E-induced responses appear to be cell- and context-specific.

PCBs and Human Exposure

PCBs cause a variety of neurological and behavioral deficits in both naturally-exposed humans and laboratory-exposed animal models. The earliest studies that examined the effects of PCBs on neurological, behavioral and developmental measures in humans took place in the 1970s after two mass outbreaks of chlorobiphenyl poisoning. In 1968, the first mass outbreak occurred in Japan when rice oil used for cooking was contaminated with Kanechlor 400, the main component of which is tetrachlorobiphenyl. Neurological examination of some of the affected individuals revealed symptoms of sensory neuropathy (e.g. numbness, pain, hypoesthesia, or areflexia of limbs) as well as reduced sensory nerve conduction velocity (Murai and Kuroiwa, 1971).

The second outbreak occurred in Taiwan in 1979 when cooking oil was contaminated with thermally degraded PCBs. Because these chemicals persist in human tissue for years and offspring of exposed females continue to be born affected, Rogan *et al.* performed a field survey of all living children who were born during or immediately after the period of contamination (Rogan *et al.*, 1988). In addition to physical abnormalities such as reduced height and weight, hyperpigmentation and an increased rate of bronchitis, the exposed children demonstrated significant delays in cognitive development. For example, the exposed children were delayed compared to controls in

the age at which they performed tasks such as saying phrases, carrying out requests, pointing to body parts or catching a ball. The exposed children also scored lower than controls on developmental and cognitive tests (i.e. the Bayley Scale of Infant Development, Stanford-Binet IQ test, and the WISC IQ test), and performed worse on the behavioral assessment test (Rutter Scale).

Examinations of children exposed to PCBs in the United States via maternal consumption of contaminated fish report similar detrimental findings. Gladen *et al.* (Gladen *et al.*, 1988) followed a cohort of children in North Carolina and found that infants 6 and 12 months of age exhibited lower psychomotor scores on the Bayley Scale after increased transplacental PCB exposure. A similar study reports hypotonicity and hyporeflexia in infants exposed to high PCB levels in breast milk (Rogan *et al.*, 1986). Jacobson *et al.* reported that Michigan children whose mothers consumed PCB-contaminated Great Lakes whitefish demonstrated various reflexive and motoric abnormalities similar to those described above in Rogan *et al.*, and that the children were also slower to accommodate to novel visual stimuli at 7 months of age (Jacobson *et al.*, 1984). When assessed at 4 years of age, these children exhibited poorer short-term memory on verbal and quantitative tests (Jacobson *et al.*, 1990). More recently, attention deficit hyperactivity disorder (ADHD) has been indicated as one of the effects of developmental exposure to PCB-like environmental contaminants (Schettler, 2001; Hardell *et al.*, 2002).

PCB-altered male fertility has caused considerable global scientific and public concern. Populations of men from Sweden (Richthoff *et al.*, 2003) to Denmark (Andersen *et al.*,

2000) to the Midwestern (Swan *et al.*, 2003) and Eastern (Hauser *et al.*, 2002) United States have experienced a decrease in sperm concentration and motility. In addition, a Swedish report revealed a strong association between the level of PCB in their mothers' blood and incidences of testicular cancer in men (Hardell *et al.*, 2003). Not only are men affected who have been directly exposed to the toxins, but a recent report has demonstrated a transgenerational effect (i.e., germline transmission to multiple generations) in males through the F4 generation after perinatal exposure of the males to endocrine disruptors (Anway *et al.*, 2005).

Additionally, while occupational exposure to PCBs has become less common, the possibility for exposure still exists for certain individuals. For example, firemen called to a fire in a Binghamton, New York, office building were exposed to PCBs after a transformer exploded and PCBs were sprayed into the air (Kilburn *et al.*, 1989). The men were exposed via contact with the skin and inhalation of the smoke and gases. All of the men reported extreme fatigue, headache, muscle weakness and aching joints, while a portion of the firefighters reported memory loss, irritability, insomnia, and an inability to concentrate.

Studying neurological and behavioral deficits in humans exposed to PCBs is challenging for a variety of reasons. The number of occurrences, lack of data on affected subjects and confounding variables are only a few of the problems associated with this type of research. In light of these difficulties, many scientists have turned to utilizing animal

models to assess the effects of PCB exposure on a variety of developmental, neurological and behavioral measures.

Using Animal Models to Evaluate the Effects of PCBs

Experimentation with animal models allows for greater flexibility than using humans to evaluate the effects of PCBs. For example, examining the effects of PCBs on developmental measures often requires that the animals be exposed to the toxins both pre- and postnatally, due to the nature of the measures that are being examined. This is accomplished by treating the female for varying lengths of time prior to and/or during pregnancy and lactation, and examining the offspring. The duration of PCB exposure can vary from a single dose of PCB during pregnancy (Faqi *et al.*, 1998) to treatment that begins prior to mating and stops at parturition (Hany *et al.*, 1999a) or continues throughout lactation (Rice and Hayward, 1999). Other experiments treat the dams for an intermediate amount of time – three days (Gupta, 2000) or 13 days (Wang *et al.*, 2002) during pregnancy. Although the above mentioned paradigms differ in length of treatment, it is important to note that because of the toxins' ability to reach a developing fetus *in utero* (Kodama and Ota, 1980) and their affinity for accumulating in adipose tissue (Heinrich-Hirsch *et al.*, 1997), the offspring of exposed mothers are likely to be directly affected by the PCB both during gestation and via lactation in all cases, albeit to different degrees.

An additional benefit of using animal models to learn about the effects of PCBs is the freedom to manipulate the type of exposure that the animals receive (i.e. the specific PCB congener(s) to which the animals are exposed). Generally, experiments can be separated into those that evaluate the effects of PCB mixtures and those that examine a single congener. One benefit of utilizing mixtures is being able to assess the effects of biologically relevant mixtures, such as those that are found in the environment (Jonsson *et al.*, 1976; Seegal *et al.*, 1991; Chung *et al.*, 2001) and in human breast milk (Lilienthal *et al.*, 2000; Kaya *et al.*, 2002). However, this method comes with its disadvantages. For example, using mixtures does not allow for isolation of the effects of specific toxins, and can lead to results in which the congeners that comprise the mixture act synergistically to produce effects that cannot be achieved when using single congeners. In some cases, the results may be synergistic and some compounds may amplify the effects of others. Thus, experiments will examine either PCB mixtures or single congeners based on the specific goals of the study that is being conducted.

Effects of PCBs Using Animal Models

Regardless of the length or type of exposure, utilizing animal models to evaluate the effects of PCBs has led to a wealth of literature revealing a variety of deficits caused by the contaminants. In rats, PCB exposure has been shown to reduce weight gain from birth to weaning in exposed offspring (Rice and Hayward, 1999), increased ano-genital distance in female (Wang *et al.*, 2002) and male (Faqi *et al.*, 1998) neonates, reduce testes weights and serum testosterone levels and increase uterine weights (Hany *et al.*,

1999b) and reduce daily sperm production (Faqi *et al.*, 1998). Neurological effects resulting from PCB exposure include increased (Seegal *et al.*, 1997; Roth-Harer *et al.*, 2001) and decreased (Seegal *et al.*, 1997; Seegal *et al.*, 1986; Seegal *et al.*, 1994; Seegal *et al.*, 1998) dopamine concentrations in the brain depending on the type of PCB that is used and brain areas that are examined, and decreased thyroid hormone levels (Seo *et al.*, 1995: Lilienthal *et al.*, 1997; Rice and Hayward, 1999).

The behavioral effects of PCB exposure are equally numerous. Monkeys exposed to PCB mixtures exhibited deficits in spatial learning and memory (Schantz *et al.*, 1991; Rice and Hayward, 1997). Experimentation with rats has revealed a feminization in sweet preference behavior in adult males (Hany *et al.*, 1999a; Kaya *et al.*, 2002), as well as an alteration in male sexual behavior (Faqi *et al.*, 1998) and female sexual behavior and receptivity (Wang *et al.*, 2002; Chung and Clemens, 1999; Chung *et al.*, 2001). PCB-exposed offspring have also showed impairments in passive avoidance behavior as adults (Weinand-Harer *et al.*, 1997). Finally, and perhaps most important for this dissertation, PCB exposure during gestation altered the display of various measures of maternal behavior in rats (Simmons *et al.*, 2005). Subcutaneous (sc) treatment with PCB 77 (3, 4, 3', 4'-tetrachlorobiphenyl) on gestation days (GD) 6 – 18 decreased the amount of time spent in high crouch nursing, altered the amount of time the dams spent on the nest, and increased pup mortality.

In addition to the concern for organisms that have been exposed directly by consumption, offspring of exposed individuals are at particular risk for being affected by maternal PCB

exposure both *in utero* and via lactation because of the toxin's ability to enter the placenta and its affinity for adipose tissue. Examining organisms that have been exposed perinatally is particularly important considering the amount of neurological and physiological development that takes place at this time.

However, exploring the effects of contaminants on individuals who have been exposed via maternal treatment is confounded by the dynamic relationship between mother and offspring. The traditional experimental paradigm used to evaluate the effects of a contaminant involves treating a pregnant animal and examining her offspring for subsequent effects. Rarely are the possibilities that the dam's exposure to the contaminant could have altered the care she provides to the offspring, or that her behavior may change when caring for affected pups. It is not unusual for a dam to adjust her behavior according to the behavior and physiology of her offspring (Stern and Keer, 2002; Stern and Johnson, 1990; Stern and Lonstein, 1996; Moore, 1982; Leigh and Hofer, 1973). A change in the dam's care can significantly affect the developing pups (Moore *et al.*, 1992; Moore, 1992; Liu *et al.*, 2000; Caldji *et al.*, 1998; Francis *et al.*, 1999) – effects that may be mistakenly attributed to the direct effects of the toxin on the offspring. It is for these reasons that maternal care must also be evaluated when examining the effects of toxin on offspring via maternal exposure.

The Importance of Maternal Behavior in the Development of the Offspring

Maternal care has a major impact on the physiological and behavioral development of the offspring. Since the 1960s scientists have noted the importance of infant stimulation and its enduring effect on offspring (Denenberg, 1969; Levine and Thoman, 1969). One of the most-studied behaviors, and arguably one of the most important behaviors, exhibited by a newly parturient dam is pup-directed licking and grooming (LG). Natural variations in LG exist among dams, with a percentage of the dams licking their offspring significantly more or less frequently than most dams. This difference is most prominent within the first postnatal week, especially on postnatal days 2 – 5 (Champagne *et al.*, 2003). A large amount of pup-directed LG is directed toward the pups' ano-genital region. This type of LG is distributed unequally between a dam's offspring with males receiving more attention than their female littermates (Moore and Morelli, 1979), and is mediated by testosterone (Moore, 1982). Pup-directed LG has been shown to be involved in multiple aspects of the offsprings' development such as "programming" their hypothalamic-pituitary-adrenal response to stress (Liu *et al.*, 1997), affecting the number of motor neurons in a sexually dimorphic nucleus of the spinal cord (Moore *et al.*, 1992), altering male sexual behavior (Moore, 1984) and female reproductive success (Gomes *et al.*, 1999), and changing gene expression by altering DNA methylation and chromatin structure in the offsprings' epigenome (Weaver *et al.*, 2004).

Taking into account the considerable role maternal care plays in the development of the offspring, the finding of Simmons and colleagues that maternal behavior is altered after

exposure to polychlorinated biphenyls has enormous implications for offspring reared by PCB-exposed dams. Further, as mentioned above, the offspring of exposed mothers are likely to be directly affected by the PCB both during gestation and via lactation. This may result in behavioral and/or physiological changes in the litter, leading to further alterations in the dam's behavior as she responds to affected pups.

Objectives

This dissertation will begin by addressing the feasibility of using an oral route of administration to examine the effects of PCB 77 on maternal behavior in Long Evans rats. Next, this paper will examine the effects of 2 mg/kg PCB 77 on various measures of maternal care and early offspring development. Subsequently an intermediate dose of PCB 77 will be examined to replicate findings and expand the behavioral analysis of the previous experiment. Finally, this dissertation will evaluate the effects of perinatal PCB exposure on offspring sexual behavior and partner preference. This paper will end with a discussion of the implications of this research with regards to the direction of future experiments and limitations of the original experimental paradigms used to evaluate the effects of contaminants on exposed offspring.

Chapter 2. Experimentation with oral administration of PCB 77

ABSTRACT

Polychlorinated biphenyls (PCBs) are environmental toxins known to affect neurobehavioral development in many laboratory studies using different animal models. Because of their bioaccumulation and long half-life PCBs are a serious concern for our own species. Recently, a study was conducted in our laboratory that examined the effects of the dioxin-like PCB congener 3, 4, 3', 4'- tetrachlorobiphenyl (PCB 77) on maternal care in rats using a subcutaneous dose of PCB during the animals' gestation period. However, since human exposure typically occurs via food consumption, we decided to examine the possibility of using an oral route of administration to investigate the effects of PCB on maternal behavior. To this end, we conducted a series of experiments to test the feasibility of treating pregnant rats orally with PCB 77. In the first experiment, pregnant rats were treated with PCB- or oil-laced vanilla wafers on gestation days (GD) 6 – 18. The majority of dams did not consume the wafer for the duration of the experiment, so the second and third experiments were conducted to test if the animals were developing a taste aversion to the wafers. In the second experiment, pregnant rats were given an untreated vanilla wafer after being injected subcutaneously with either oil or PCB 77 in the dose of 2 or 4 mg/kg. There was no significant effect of treatment on cookie consumption in any of the groups. Thus, subcutaneous administration of the PCB did not support the development of a taste aversion to the wafer. In the third experiment non-pregnant rats were given one-half of a vanilla wafer treated as in the first experiment

with either oil or 2 or 4 mg/kg PCB 77 emulsified in oil. The animals in the oil group consumed all or the majority of each cookie. The animals in the 2 mg/kg group began to eat the cookies at the start of the experiment but stopped within a week, whereas the females in the 4 mg/kg group never ate the cookies. Overall, the results indicate that voluntary oral administration of PCB 77 is not an option for the study of effects of this contaminant on maternal behavior, since as the animals develop an aversion to the cookie, they discontinue or reduce their exposure to the PCB.

INTRODUCTION

Previously, the effects of PCB on maternal behavior were examined by our group after subcutaneous (sc) injection of PCB 77 (Simmons *et al.*, 2005). However, PCB exposure in humans occurs during food consumption as a result of the toxin's accumulation in the food chain, and it is a concern whether the effects with sc administration mirror those that are experienced after oral ingestion. Therefore, it is important to explore the possibility of studying the effects of PCB 77 on maternal behavior using an oral route of administration.

Different methods have been employed to administer PCB orally to animals. A number of experiments have used a gavage technique, in which the PCB is emulsified in oil and force-fed to the animal through a tube inserted orally into the animal's esophagus (Crofton *et al.*, 2000; Agrawal *et al.*, 1981; Widholm *et al.*, 2001; Faqi *et al.*, 1998; Schantz *et al.*, 1996). However, this method may put the animal under a great deal of

stress, which could also significantly affect maternal behavior (Moore and Power, 1986; Smith *et al.*, 2004) or modify the physiology and/or behavior of the pups that were exposed to the stress *in utero* (Ward and Weisz, 1980; Vom Saal *et al.*, 1990; Griffin *et al.*, 2003; Weinstock, 1997; Takahashi *et al.*, 1992). Experiments have also presented the toxin by lacing the animals' daily food source with the PCB (Hany *et al.*, 1999a; Kaya *et al.*, 2002) or by adding the oil-emulsified PCB to cookies or treats that were given to the animals (Seegal *et al.*, 1997; Rice and Hayward, 1999). Although both methods avoid the stress of gavage, it is often difficult to be sure that each animal receives equivalent doses of PCB when treating the animals' food source with the toxin if all of the food is not consumed. This becomes particularly problematic when investigating contaminants that have estrogenic actions since estradiol is known to reduce food intake (see Wade and Gray, 1979 for review).

In the interest of paralleling the route of exposure that occurs naturally in the human we conducted a pilot study to determine the feasibility of using an oral route of administration to evaluate the effects of PCB 77 on maternal care in rats. To this end we followed procedures reported by Seegal *et al.* (1997) with slight modifications and presented pregnant rats with cookies adulterated with PCB.

Experiment One: PCB Cookies, A Pilot Study

EXPERIMENTAL DESIGN

In order to compare the results of the current study with our earlier one that used sc PCB administration (Simmons *et al.*, 2005) the dams in this pilot study were treated on the same gestation days (GDs) with the same doses of PCB. Thus, pregnant rats were fed one-half of a vanilla wafer cookie treated with the corn oil vehicle, 2 mg/kg or 4 mg/kg PCB 77 emulsified in corn oil on GDs 6 – 18. There were four dams in each treatment group. Cookies were prepared the day before by dispensing the appropriate amount of corn oil (with or without PCB) on to the wafers and placing them in the fume hood overnight to allow for absorption of the oil/PCB mixture.

RESULTS

Early into the experiment, we noticed a few things. First, two of the twelve dams (both from the 4 mg/kg group) never appeared to eat any of the cookies. The animals that did eat the cookies (the remaining 10 animals) began doing so after one or two presentations. Finally, regardless of dose, all the PCB-treated dams that began eating the cookies stopped after a week. Thus, the only dams that consumed the cookies for the duration of the experiment were those in the oil group.

DISCUSSION

It is unclear why our outcome differed from that of Seegal *et al.* (1997), the study from which we derived our methods. However, there were some significant methodological differences. Most notably is that the two studies used different strains of rat. Seegal et al treated pregnant Sprague-Dawley rats with PCB while we used Long Evans. These two strains exhibit a long history of significant differences for a variety of measures including likelihood of nicotine self-administration (Shoaib *et al.*, 1997), physiological responses to stress (Faraday *et al.*, 2005) and length of time required prior to pup sensitization (Stern, 1989), to name a few. In addition, while both strains of rat are considered to be susceptible to the toxic effects of dioxin, the Long Evans strain is known to be the most vulnerable (Long Evans, LD50 ~ 10 μg/kg; Sprague-Dawley, LD50 ~ 50 μg/kg; Franc *et al.*, 2001). Long Evans rats are often used when the purpose of an experiment is to assess the effects of dioxin on a strain of rat that is highly susceptible to TCDD (Unkila *et al.*, 1994; Pohjanvirta *et al.*, 1988; Pohjanvirta *et al.*, 1993; Huuskonen *et al.*, 1994). Thus, since PCB 77 is a dioxin-like congener, it is not surprising that pregnant rats from these two groups responded differently to ingestion of the PCB. Additionally, Seegal *et al.* used a lower dose of PCB 77 (0.1 or 1 mg/kg) over a longer period of time, and adjusted the dose of PCB that was applied to the cookie according to weight twice weekly, as opposed to every day as was done in our experiment. This may have decreased the actual amount of PCB the dams were consuming from the intended 1 mg/kg to a lower dose.

We were not surprised that many of the dams did not eat the cookie until the second or third day as rats are extremely neophobic (Richter, 1953; Barnett, 1956) and will often only sample a novel food item when it is first presented (Rzoska, 1953) so that they can "test" the safety of the novel food. What we did find unusual, however, was that after the dams appeared to overcome their neophobia and ate the cookies, many stopped after a few days. These events suggested to us that the dams might have fallen ill as a result of the ingested PCB and developed a taste aversion to the cookies. A learned taste aversion occurs when an animal associates the flavor of something it has eaten with an unpleasant effect that occurs after consumption of the item, although the food may not have been what directly caused the adverse effects. If consuming the PCB caused the dams to become ill, they may have associated the sickness with the consumption of the cookie. A taste aversion can often be learned in as little one trial (Garcia *et al.*, 1966), but less toxic stimuli may require up to four trials to be effective (Gomez, 2001). Thus, the dams could have developed a taste aversion to the PCB-treated cookies if exposure to the toxin caused them to become ill and they associated the sickness and the novel food.

To further investigate this hypothesis a second experiment with pregnant rats receiving sc injections of PCB 77 was conducted.

Experiment Two: Subcutaneous Administration of PCB 77 to Pregnant Rats and Consumption of an Untreated Cookie

The dams in the previous study may have stopped eating the vanilla wafers because they became ill as a result of ingesting the PCB. However, they may have become sick simply as a result of the presence of the toxin in their bodies, regardless of the route of administration. If the dams became sick as a result of the presence of PCB, then we would expect that dams receiving PCB sc should also become ill. If these dams are given oil-treated vanilla wafers at the time of PCB injection, they may associate negative effects of PCB exposure (e.g. becoming sick) with the novel food item and stop eating it. On the other hand, if oral delivery of the toxin is required for the dams to become ill, then sc injection will not cause the dams to become sick and they should continue to eat the oil-treated cookie. In order to determine if the presence of PCB in the body will cause the dams to become sick and develop a taste aversion to a novel food item, we treated pregnant rats with PCB sc, fed them an oil-treated vanilla wafer, and monitored their cookie consumption. Thus, we used the development of a taste aversion as a way to gauge whether the dams experienced negative side effects of PCB exposure, such as becoming ill.

METHODS

Animals and Housing

Fourteen timed-pregnant Long-Evans rats from Charles River Laboratories (Raleigh, North Carolina) arrived at our laboratory on GD 5 and were housed singly in plastic cages (45 X 22 X 21 cm) with wood shavings as bedding in a laminar-flow unit (NUAIRE). Animals were maintained on a 14:10-h light:dark cycle with lights off at 1100 h and a dim red light on at all times. The ambient temperature was 21 +/- 1°C. The rats were allowed free access to water and Harlan Rodent Chow 8640 food pellets. Our animal facilities are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, and the Michigan State University Animal Care and Use Committee approved experimental procedures.

Procedure

The dams were weighed daily and given subcutaneous injections of the oil vehicle or PCB 77 emulsified in corn oil in the dose of 2 or 4 mg/kg from GD 6 to GD 18. At this time, dams were also given a half of a vanilla wafer onto which corn oil had been dispensed in accordance with their weight from the previous day. Cookies were weighed just prior to being placed in the nest and again after the testing period if any piece(s) remained. The female was allowed 10 min to consume the cookie, which had been shown in a previous experiment to be plenty of time for the animal to eat the entire wafer

(Rice and Hayward, 1999). The number of dams in each group was oil = 4, 2 mg/kg = 6, 4 mg/kg = 4. Nest building materials in the form of thirty brown paper towel strips were placed on the wire top of each cage on GD 20. Parturition took place during the mid- to late-light phase of the animals' light/dark cycle on GD 22 - 23.

RESULTS

The 3 X 13 ANOVA (treatments X days, with repeated measures on the second factor) analyzing the effect of subcutaneous PCB treatment on cookie consumption did not detect any significant main effects or interactions (Figure 1).

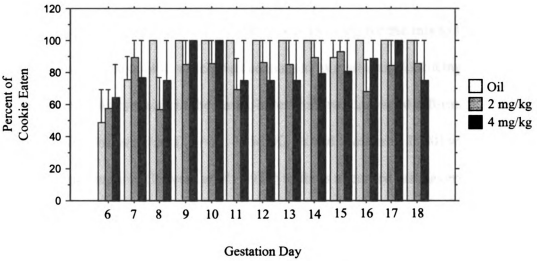


Figure 1. The percent of oil-treated cookies eaten by pregnant rats on gestation days 6 – 18. The ANOVA did not detect any significant main effects of subcutaneous PCB treatment or interactions.

DISCUSSION

Feeding oil-treated cookies to pregnant rats at the time of sc PCB administration did not cause the dams to stop eating the wafers. Thus, either sc administration of the toxin did not cause the dams to become ill, or the length of time between the effects of sc injection of the PCB and the ingestion of the cookies was too long for the rats to form a learned taste aversion to the cookie which was consumed immediately following the injection. Vagal sensory fibers reside in the gastro-intestinal tract and send information to the nucleus of the solitary tract (Torvik, 1956; Kalia and Mesulam, 1980) where it converges with taste information (Travers *et al.*, 1987) to form a learned taste aversion. When the toxin is injected, then perhaps the timing of convergence of information after tasting the cookie and experiencing any putative negative effects of the PCB treatment is not conducive to the development of a taste aversion, as is the case when the cookie and the PCB are ingested together. The animal may become ill as a result of the PCB injection, but may not recognize the cookie as the source of the illness if the length of time between ingesting the cookie and feeling sick is too great. Alternatively, although the eventual fate of PCB in the body is probably similar (i.e. accumulation in adipose tissue), after different routes of administration the final concentration of each metabolite and what chemical changes the toxin undergoes prior to being fully metabolized are likely to be very different with oral and sc administration. This differential metabolism may account for the different results seen with the two routes of administration examined. These two hypotheses are not mutually exclusive.

Experiment Three: Oral Administration of PCB 77 to non-pregnant rats

Pregnant rats that were given oil-treated vanilla wafers at the time of sc PCB administration did not develop a taste aversion, that is, the females did not stop eating the cookies. As mentioned above, sc PCB administration either does not cause dams to become physically ill, or the length of time between tasting the cookie and experiencing the effects of the toxin are too great for the animal to make the association. It is also possible that the effect we saw in the pilot experiment (that the dams stopped eating the PCB-treated vanilla wafers) was due in part to the pregnant state of the females. Estrogen has been known to modulate learning (Leuner *et al.*, 2004) including the rate of extinction of conditioned taste aversions (Yuan and Chambers, 1999). Estrogen levels in female rats are relatively low early in the pregnancy (30 pg/ml; Bridges, 1984) but can reach 110 pg/ml during the proestrus phase of the estrous cycle (Schwartz, 1974; Nequin *et al.*, 1975) in a cycling female. This may cause the pregnant and non-pregnant rats to respond differently to the PCB-treated cookie, resulting in the non-pregnant females eating the PCB-laced cookies even though their pregnant counterparts did not. To this end, we examined the effects of PCB consumption in non-pregnant females to see if these animals would react in the same way to the ingested toxin and develop a similar taste aversion to the PCB-treated wafers. Thus, our next experiment involved treating non-pregnant females with PCB-laced cookies.

METHODS

Animals and Housing

Fourteen 60-day-old Long-Evans rats from Charles River Laboratories arrived at our laboratory and were housed according to the Methods described above in the Pilot Experiment.

Procedure

The day the females arrived at our laboratory was denoted day 0 with treatment beginning on day 1. This was done in order to keep as close as possible to the treatment received by the pregnant rats in Experiments One and Two, which arrived at our laboratory on GD 5 and began treatment on GD 6. Females were randomly assigned to one of three different groups and received one half of a vanilla wafer on days 1 – 13 that was treated with the PCB or oil according to her weight the previous day, as described above in the Pilot Experiment. One group received a cookie treated only with the corn oil vehicle, one with 2 mg/kg PCB 77 and one with 4 mg/kg PCB 77 emulsified in corn oil. The number of animals in each group was oil = 4, 2 mg/kg = 6, 4 mg/kg = 4.

Cookies were also weighed just prior to being placed in the cage, as described above in Experiment One. Most animals that ate the cookie did so immediately. Animals that did

not consume the cookie usually buried it in the bedding in a corner or sniffed it and walked away. Cages were searched after the 10 min were up and any remaining pieces of the cookie were weighed. The percentage of cookie that was consumed was calculated and recorded.

RESULTS

Using a 3 X 13 ANOVA to analyze cookie consumption (treatments X days, with repeated measures on the second factor) we detected a significant interaction between PCB treatments and days [F (24, 132) = 8.37, $p < 0.0001$; Figure 2]. Further analysis did not reveal a simple effect of treatment on days 1 and 2, but it did on days 3 – 13. For days 3 – 13, the simple effect of treatment was evaluated using Bonferroni t-tests for independent samples with the significance level held at .05/3, or $P < 0.017$.

Every day on which the oil group was significantly different from a PCB group, the oil group ate more of the cookie. On days 3 and 4, the groups differed in a dose-dependant fashion, with the oil group eating more than the other two groups, and the 2 mg/kg group eating more than the 4 mg/kg group. On the rest of the treatment days, the oil group differed from the 2 and 4 mg/kg groups, with the females in the oil group eating more of the cookies than the other two groups (See Table 1 for a summary of the comparisons of treatment means for each day).

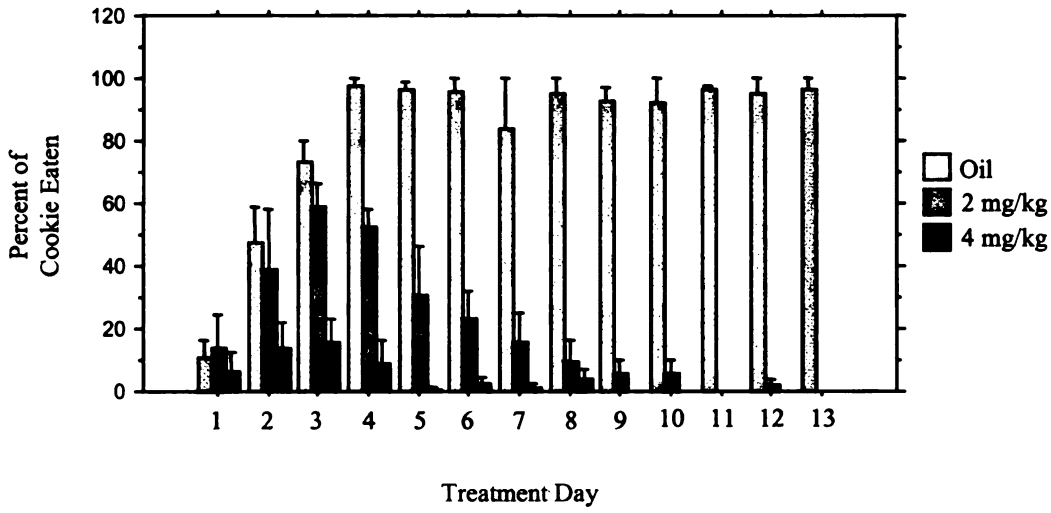


Figure 2. The percent of oil- or PCB-treated cookies eaten by non-pregnant rats on treatment days 1 – 13. The ANOVA detected a significant interaction between PCB treatments and days, and further analysis revealed a simple effect of treatment on days 3 – 13.

TABLE 1

Comparisons of treatment groups for the amount of PCB-laced cookie consumed by day

Day	Group Differences in Cookie Consumption
1	Not significant
2	Not significant
3	oil > 4 mg ($t = 5.68$, $df = 6$, $p = 0.001$)
	2 mg > 4 mg ($t = 3.80$, $df = 8$, $p = 0.005$)
4	oil > 2 mg ($t = 6.12$, $df = 8$, $p = 0.001$)
	oil > 4 mg ($t = 10.75$, $df = 6$, $p < 0.001$)
	2 mg > 4 mg ($t = 4.68$, $df = 8$, $p < 0.002$)
5	oil > 2 mg ($t = 3.36$, $df = 8$, $p = 0.010$)
	oil > 4 mg ($t = 36.24$, $df = 6$, $p < 0.001$)
6	oil > 2 mg ($t = 6.51$, $df = 8$, $p < 0.001$)
	oil > 4 mg ($t = 20.91$, $df = 6$, $p < 0.001$)
7	oil > 2 mg ($t = 3.91$, $df = 8$, $p = 0.005$)
	oil > 4 mg ($t = 5.16$, $df = 6$, $p = 0.002$)
8	oil > 2 mg ($t = 8.95$, $df = 8$, $p < 0.001$)
	oil > 4 mg ($t = 14.46$, $df = 6$, $p < 0.001$)
9	oil > 2 mg ($t = 15.60$, $df = 8$, $p < 0.001$)
	oil > 4 mg ($t = 21.27$, $df = 6$, $p < 0.001$)
10	oil > 2 mg ($t = 10.25$, $df = 8$, $p < 0.001$)
	oil > 4 mg ($t = 11.50$, $df = 6$, $p < 0.001$)
11	oil > 2 mg ($t = 92.59$, $df = 8$, $p < 0.001$)
	oil > 4 mg ($t = 73.20$, $df = 6$, $p < 0.001$)
12	oil > 2 mg ($t = 19.14$, $df = 8$, $p < 0.001$)
	oil > 4 mg ($t = 18.05$, $df = 6$, $p < 0.001$)
13	oil > 2 mg ($t = 30.36$, $df = 8$, $p < 0.001$)
	oil > 4 mg ($t = 24.00$, $df = 6$, $p < 0.001$)

DISCUSSION

Ingestion of PCB cookies by non-pregnant rats

One of the purposes for conducting Experiment Three was to determine if the dams in the first experiment stopped eating the cookies because of an interaction between the pregnancy and the ingested PCB. The data from this experiment indicate that this was not the case. The non-pregnant females in Experiment Three that received cookies adulterated with 2 mg/kg PCB also stopped eating them after a few days. Thus, the fact that the pregnant dams in the first experiment stopped eating the cookies does not appear to be related to their state of pregnancy (i.e. decreased estrogen levels or elevated levels of progesterone).

Again, it is unclear why the animals in our experiment stopped eating the PCB-treated cookies and those in Seegal *et al.* (1997) did not. As mentioned in the Discussion section of the Pilot Experiment, they used the same PCB congener that we do here, although at a lower dose (1 mg/kg/day) but over a longer period (GD 6 through weaning), and the most likely reason for our conflicting results is that we used a different strain of rat than Seegal *et al.*

Interestingly, the 2 and 4 mg/kg doses of PCB evoked different reactions. Although dams from each of the three treatment groups (oil, 2 mg/kg and 4 mg/kg) exhibited a species-typical neophobia at the beginning of the experiment and ate very little of their

cookies, the oil and 2 mg/kg groups ate significantly more of their cookies than the 4 mg/kg group on the third and fourth days. From the fifth day onward, both PCB-treated groups ate significantly less of their cookies than the oil group. While the females in the lower PCB group continued to eat the cookie for a few days before reducing the amount that they consumed and eventually stopping altogether, the 4 mg/kg group ate very little from the start and even after repeated presentations of the cookies.

The data obtained for the 4 mg/kg group are consistent with two different hypotheses. First, it is possible that the 4 mg/kg PCB that was dissolved in oil and applied to the cookie was in a concentration too high to remain fully emulsified in the oil, leaving an unfavorable tasting residue on the cookie. Alternatively, there is a chance that the amount of PCB ingested from the sampling of the cookie was enough to cause the females to experience adverse physical side effects and develop a taste aversion. The 4 mg/kg dose has been shown to decrease the viability of offspring and interfere with delivery of the litter when administered sc, indicating a high degree of toxicity (Simmons *et al.*, 2005). However, those effects were exhibited after the full treatment of PCB over 13 gestation days. Therefore, it seems unlikely that the small amount of PCB consumed by the females in the 4 mg/kg group would result in such a strong negative reaction and cause the development of a taste aversion, indicating that the females in this group likely stopped eating the adulterated wafers because of an unpleasant taste of the PCB. But, our data do not allow us to differentiate between these two competing hypotheses at this time.

General Conclusions

For Long Evans rats, the use of a laced cookie to administer PCB 77 does not seem to be a viable approach at least for the doses of interest for the research of this dissertation. The data indicate that there are complex interactions between the dose of PCB and the route of administration used to deliver the toxin (e.g. compare the results of the Pilot Experiment and Experiment 2), but the systematic investigation of those interactions is outside the scope of this project. Since forced ingestion of the PCB using the gavage technique is clearly too stressful to use with pregnant animals (see Introduction), the remainder of the experiments in this dissertation will focus on evaluating the effects of PCB on maternal behavior using subcutaneous administration of the toxin in order to ensure that the animals are receiving an accurate dose of PCB and to minimize stress experienced by the dam. Previous experiments have successfully used a subcutaneous route of administration to examine the effects of PCB on a variety of measures (Hany *et al.*, 1999a; Hany *et al.*, 1999b; Weinand-Harer *et al.*, 1997; Lilienthal *et al.*, 1997; Roth-Harer *et al.*, 2001). In addition, Hany *et al.* (1999b) have shown that PCB 77 accumulates in brain and adipose tissue of dams and offspring after sc maternal exposure.

Chapter 3. How does PCB 77 (2 mg/kg b.w.) on gestation days 6 – 18 affect the maternal care of a rat when utilizing a cross-fostering design?

INTRODUCTION

Polychlorinated biphenyls (PCBs) are persistent contaminants that remain in the environment because of their stable chemical structure and long half-life. Although they were banned from use in the 1970s, PCBs can still be found today in sediment, run-off and wildlife, and have also been detected in human umbilical cord plasma and breast milk (Vreugdenhil *et al.*, 2002; Mes and Marchand, 1987; Noren *et al.*, 1990; Noren and Lunden, 1991; Duarte-Davidson *et al.*, 1992; Gonzalez *et al.*, 1995). These findings indicate a risk not only to adult individuals that are exposed to the toxicants, but also to the more vulnerable developing organism exposed *in utero* and via lactation. As discussed in the Introduction to this dissertation, studies examining humans that have come in contact with PCBs (Rogan *et al.*, 1988; Gladen *et al.*, 1988; Dewailly *et al.*, 1991) and laboratory studies with various animal models (Hany *et al.*, 1999a; Hany *et al.*, 1999b; Faqi *et al.*, 1998; Seegal *et al.*, 1997; Schantz and Widholm, 2001) have demonstrated that PCB exposure is associated with both neurological and behavioral deficits.

In the present experiment we focus on the dioxin-like PCB congener 3, 4, 3', 4'-tetrachlorobiphenyl (PCB 77) because it has high toxic potency and affinity for the Ah receptor, has a low degradation rate *in vivo*, and it is among the most abundant PCB

congeners in human tissues (Safe, 1994). Further, *in vivo* and *in vitro* studies have shown that PCB 77 has both estrogenic and anti-estrogenic activity (Seegal *et al.*, 2005; Garner *et al.*, 1999; Nesaretnam *et al.*, 1996; Jansen *et al.*, 1993; and see Hansen, 1998 for review). PCB 77 is detected in the brains of the developing offspring as well as in the brains of the dams when administered to rats subcutaneously during pregnancy (Hany *et al.*, 1999b) thus, it could affect the central nervous system and behavior of both the developing litter and the pregnant adult. Developmental effects of PCB 77 on the behavior of male and female offspring are well-documented (Faqi *et al.*, 1998; Chung *et al.*, 2001; Wang *et al.*, 2002), and recently we reported an effect of this contaminant on the maternal behavior of rats (Simmons *et al.*, 2005). In addition to its possible estrogenic and anti-estrogenic actions, under some conditions PCB 77 exposure enhances central dopaminergic activity (Seegal *et al.*, 1997; Seegal *et al.*, 2005), and both estrogen and dopamine play significant roles in several aspects of maternal behavior in rats (Fahrbach *et al.*, 1985; Stern and Taylor, 1991). Recently, Seegal *et al.* (1995) have shown that PCB 77 can increase prefrontal cortex DA by acting estrogenically, demonstrating a direct link between PCB 77, estrogen and DA.

The few studies that have reported effects of environmental contaminants on maternal behavior of mammals (mice, Palanza *et al.*, 2002a; Palanza *et al.*, 2002b; rats, Simmons *et al.*, 2005) have used experimental protocols in which both the pregnant animal and the developing litter are exposed to the toxic agent. With that approach it is impossible to differentiate between effects on maternal behavior that are the result of direct actions on the dams' endocrine and/or nervous systems from those that stem from indirect effects

mediated by changes in the physical and behavioral profiles of the exposed litter. Here we use a cross-fostering design in an attempt to gain insights about the contributions of direct and indirect effects of PCB 77 exposure on maternal behavior in rats. We also investigated if the maternal behavior of dams that were exposed to PCB, or who raised pups exposed to PCB, was different from that of dams that were never exposed to the contaminant and raised unexposed litters.

METHODS

Procedure

The dams were weighed daily and given subcutaneous injections of the oil vehicle or 3, 4, 3', 4'-tetrachlorobiphenyl (PCB 77; Accustandard, New Haven, CT; >99% pure as determined by gas chromatography performed by the vendor) emulsified in corn oil in the dose of 2 mg/kg from GD 6 to GD 18. Selection of this dose and treatment period was based on the findings that a dose of 1.5 mg/kg PCB 77 on GD 7-18 resulted in detectable but relatively low concentration of the contaminant in the maternal nervous system (Hany *et al.*, 1999b), as well as on the observation that the higher dose used here affects the maternal behavior of dams treated during this gestational period (Simmons *et al.*, 2005). Nest building materials in the form of thirty brown paper towel strips were placed on the wire top of each cage on GD 20. Parturition took place during the mid- to late-light phase of the animals' light/dark cycle on GD 22 - 23. On the day of birth [day of birth = postnatal day (PND) 0], the litters were culled to 8 animals (4 females and 4 males) and

cross-fostered or left with their own mothers to create the following groups: (1) oil treated dams with their own oil pups, (2) oil treated dams with a new litter of oil pups, (3) PCB treated dams with their own PCB pups, (4) PCB treated dams with a different litter of PCB-exposed pups, (5) PCB treated dams with a litter of oil pups, and (6) oil treated dams with a litter of PCB-exposed pups. Each group contained six dams.

Dams and litters were videotaped on PND 1, 2, 4 and 6 for the animals' last hour of light and first hour of dark (2 hr/day/animal). Litters were weighed every day after taping. Videos were analyzed using The Observer 3.0 (Noldus Information Technology), a behavioral data acquisition computer program. During review of the videotapes, the following behaviors were recorded: time on the nest, licking and grooming of the pups, maternal autogrooming, and amount of time spent nursing. Nursing behaviors included resting, or supine nursing, in which the dam was lying on the pups or on her side, and two quiescent nursing postures: low and high crouch. The low-crouch nursing posture is one in which the dam becomes immobile in response to stimulation from the pups as they ventrally root in order to attach to a nipple prior to nursing. High-crouch nursing can be distinguished by a distinct splaying rigidity of the limbs and increased dorsal arching of the dam's back, a position that is exhibited in response to persistent stimulation from the pups, such as continued rooting and nipple attachment. Total time spent nursing is the total of all time spent in one of the three nursing postures while on the nest. The number of nursing bouts, defined as visits to the nest that involved nursing of the litter, was also recorded. Average pup weight gain and pup survival between PND 1 and PND 6 were determined for all litters. All percentages are relative to the two-hour scoring period,

save the percentages for the high crouch nursing behavior, which was calculated from the total time spent nursing.

Two investigators scored the maternal behaviors from the video recordings. Because of procedural demands, it was impossible to keep one of these two investigators unaware of the treatment received by each dam. A subset of the recordings was scored independently by both investigators and a correlational analysis was used to evaluate the inter-observer reliability. For all the maternal behaviors analyzed, the positive correlation between the two data sets was greater than .90.

Analyses

A preliminary analysis revealed that the maternal care exhibited by dams rearing adopted young of the same treatment did not differ from dams rearing their own young, regardless of whether the dam was oil or PCB treated (*t*-tests, *p*s = 0.12 - 0.94 for all behaviors examined). For this reason, data from the group of PCB mothers rearing their own PCB pups (uncrossed) were combined with those from the group of PCB dams rearing a litter of PCB pups that were not her own (crossed) resulting in a total of twelve dams and litters in this group. The same was done for the crossed and uncrossed oil groups. The remaining four treatment groups contained six dams each.

For each behavioral measure, a *t*-test was used to test the hypothesis that exposure to PCB by the dam and/or the litter affects the dams' behavior. This was evaluated by

comparing the oil group to a combined PCB group that included all groups receiving any PCB exposure. In addition, 4 X 4 ANOVAs (treatment groups X days) with repeated measures on the second factor were used to analyze the data. Significant overall *F* ratios were followed by post-hoc comparisons of the individual group means using the Fisher's PLSD test. The data on pup survival and weight gain of the litters were analyzed using *t*-tests (oil vs. all PCB groups combined) and one-way ANOVAs, followed by appropriate post hoc comparisons. The relationships between pup grooming and nursing bouts, as well as number of pups and amount of maternal autogrooming, were examined using correlational analyses (Pearson Product Moment). All differences considered statistically significant have $p \leq 0.05$.

RESULTS

A comparison of all PCB-exposed groups to the oil-only group showed that PCB exposure decreased the number of pups present on PND 6 ($t = -2.68$, df = 29, $p < 0.05$; Figure 3, Top). A one-way ANOVA of the four different treatment groups also detected a significant [$F(3, 27) = 4.37$, $p < 0.05$] treatment effect on pup survival (Figure 3, Bottom). Post hoc tests demonstrated that the main effect of treatment was due to a higher mortality for pups that had received PCB exposure *in utero*, regardless of their postnatal condition. Figure 4 summarizes the data for average weight gained by the pups

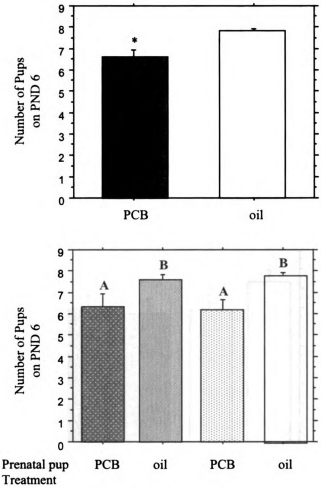


FIGURE 3. Top: A comparison of the oil-only group against the combined PCB group found a significant difference (*) in the number of pups present on PND 6, with fewer pups surviving in the combined PCB group. Bottom: A comparison of the four treatment groups using a one-way ANOVA revealed a main effect of treatment on pup survival; the groups sharing the same letter are not significantly different from each other. For this and all the other figures see the text for details of statistical analyses.

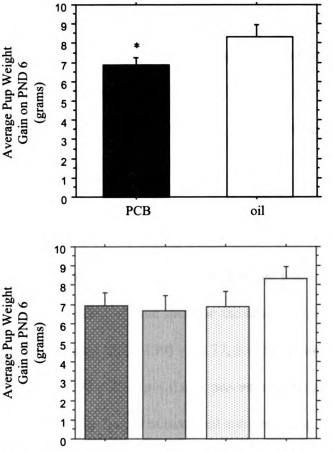
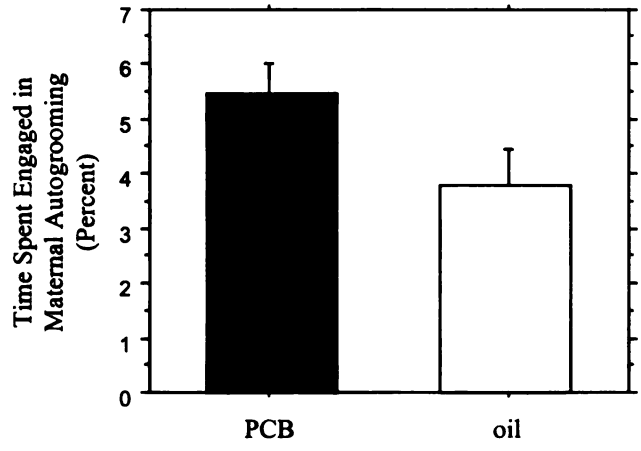


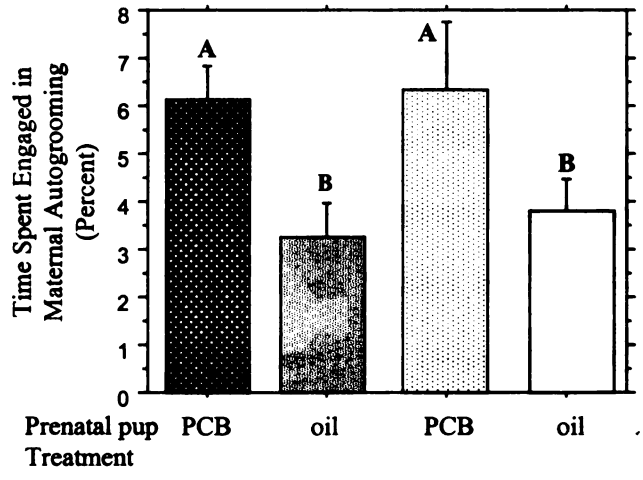
FIGURE 4. Top: A comparison of the oil-only group against the combined PCB group found a significant difference (*) in the average amount of weight that the pups gained since PND 0, with less weight gained by the combined PCB group. Bottom: The ANOVA of the data from the four treatment groups did not find a significant main effect of treatment for average amount of weight gained by the pups.

(weight gain of the litter divided by number of surviving pups) between PND 1 and PND 6. The t-test detected a significant effect of treatment (t = -2.06, df = 29, $p < 0.05$) when comparing all PCB-treated groups to the oil only group, in that the combined PCB groups gained less weight than the oil group (Figure 4, Top). Considering the four treatment groups (Figure 4, Bottom), the ANOVA did not find a significant main effect of treatment on this measure ($p = 0.28$).

For all the other dependent variables, the 4 X 4 ANOVAs found no significant interactions between treatments and days. A significant main effect of days (data not shown) was found for percent of time spent on the nest [F (3, 139) = 41.02, $p < 0.05$], percent of total time nursing [F (3, 139) = 38.77, $p < 0.05$], and in the high crouch nursing posture [F (3, 139) = 3.35, $p < 0.05$], percent of time grooming the pups [F (3, 139) = 4.05, $p < 0.05$] and engaged in maternal autogrooming [F (3, 139) = 8.29, $p < 0.05$]. Post hoc analyses showed that the amount of time spent on the nest, nursing, pup grooming and autogrooming was significantly higher on PND 1 than on PND 2, 4 and 6. The amount of time spent engaged in high crouch nursing was significantly higher on PND 6 than on PND 1 and 2. A significant effect of days was not found for the number of nursing bouts.

The t-test failed to reach significance for the amount of time spent autogrooming ($p = 0.07$; Figure 5, Top). The analysis of the four separate treatment groups identified a significant main effect of treatment on this measure [F (3, 140) = 3.28, $p < 0.05$]. Post hoc comparisons showed that dams rearing pups that were prenatally exposed to PCB





Prenatal pup Treatment	PCB	oil	PCB	oil
(Treatment of biological dam)				
Postnatal pup Treatment	PCB	PCB	oil	oil
(Treatment of dam rearing litter)				

FIGURE 5. Top: The difference between percent of time engaged in maternal autogrooming between the oil-only group and the combined PCB group missed statistical significance ($p = 0.06$). Bottom: The ANOVA of the data from the four treatment groups found a significant main effect of treatment on maternal autogrooming; the groups sharing the same letter are not significantly different from each other.

groomed themselves more than dams rearing prenatally oil-exposed pups (Figure 5, Bottom). Correlational analysis revealed a significant negative correlation (r = -0.47, df = 31, $p < 0.05$) between the number of pups present and the amount of autogrooming exhibited by the dam on PND 6.

Figure 6 shows the data on pup grooming. A significant increase in pup grooming due to PCB exposure was detected by comparing the mean of all PCB-treated groups with that of the oil-only group (t = 2.52, df = 142, $p < 0.05$; Figure 6, Top). When the four separate treatment groups were compared, the main effect of treatment missed the significance level (p = 0.08; Figure 6, Bottom). The number of nursing bouts was significantly higher when the combined PCB groups were compared the oil-only group (t = 2.02, df = 142, $p < 0.05$; Figure 7, Top). Further, the ANOVA detected a significant main effect of treatment on this measure [F (3, 140) = 3.84, $p < 0.05$], and post hoc comparisons showed that the main effect was due primarily to an increase in number of nursing bouts in the dams rearing pups that were exposed to PCB prenatally. There was no significant correlation between number of nursing bouts and amount of time spent licking and grooming the pups for the data from the PCB groups (r = 0.10, df = 96, p = 0.34) or for the oil-only group (r = 0.12, df = 48, p = 0.41).

None of the tests detected significant effects of PCB exposure on the amount of time the dams spent nursing (data not shown), but the PCB-exposed groups displayed the high crouch nursing posture significantly less than the oil-only group (t = -2.06, df = 142, $p <$ 0.05; Figure 8, Top). The ANOVA of the four treatment groups found a significant main

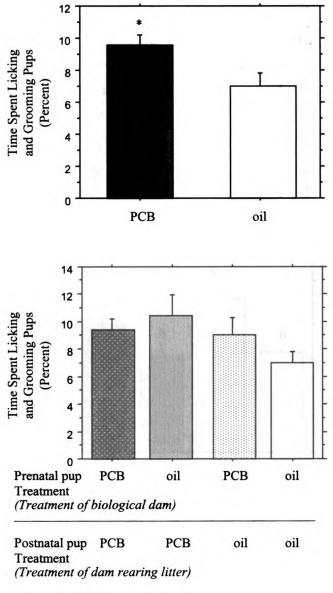
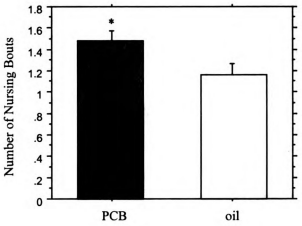
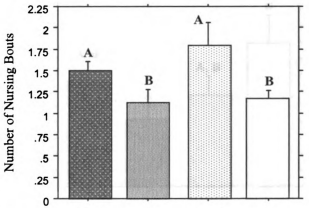


FIGURE 6. Top: A comparison of the oil-only group against the combined PCB group found a significant difference (*) in the amount of licking and grooming directed to the litter, with enhanced pup grooming seen in the combined PCB group. Bottom: The ANOVA of the data from the four treatment groups did not find a significant main effect of treatment ($p = .08$).





Prenatal pup Treatment *(Treatment of biological dam)*	PCB	oil	PCB	oil
Postnatal pup Treatment *(Treatment of dam rearing litter)*	PCB	PCB	oil	oil

FIGURE 7. Top: A comparison of the oil-only group against the combined PCB group found a significant difference (*) in the number of nursing bouts displayed by the dams, with more bouts seen in the combined PCB group. Bottom: The ANOVA of the data from the four treatment groups found a significant main effect of treatment on the number of nursing bouts; the groups sharing the same letter are not significantly different from each other.

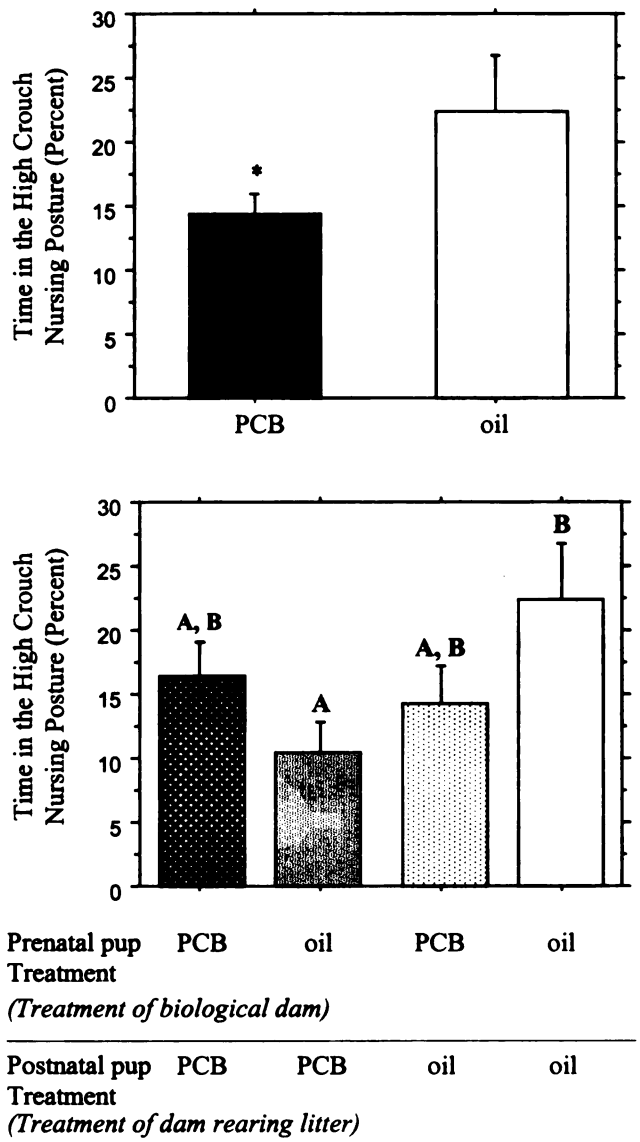


FIGURE 8. Top: A comparison of the oil-only group against the combined PCB group found a significant difference (*) in the display of the high crouch nursing position, with the PCB group showing less high crouch nursing. Bottom: The ANOVA of the data from the four treatment groups found a significant main effect of treatment on the display of high-crouch nursing; the groups sharing the same letter are not significantly different from each other.

effect of treatment for the amount of time that the dams displayed the high crouch nursing posture [F (3, 140) = 1.81, $p < 0.05$], and post hoc tests found a significant difference between the oil-only group and the group with PCB-exposed dams rearing oil-treated pups (Figure 8, Bottom).

The t-test showed that the PCB groups spent significantly more time on the nest when compared to the oil-only group (t = 1.99, df = 142, $p < 0.05$; Figure 9, Top). The ANOVA of the four treatment groups found a significant main effect of treatment for time spent on the nest [F (3, 140) = 1.94, $p < 0.05$; Figure 9, Bottom]. The three groups exposed to PCB tended to show more time on the nest, but post hoc analysis found a significant difference only between the oil-only group and the group with PCB-exposed dams rearing pups prenatally exposed to PCB.

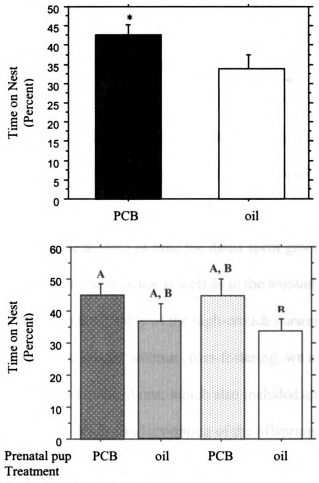


FIGURE 9. Top: A comparison of the oil-only group against the combined PCB group found a significant difference (*) in the amount of time the dam spent on the nest, with more time on the nest seen in the combined PCB group. Bottom: The ANOVA of the data from the four treatment groups found a significant main effect of treatment on time spent on the nest; the groups sharing the same letter are not significantly different from each other.

DISCUSSION

Comparison of PCB Treatments with Controls

Various components of maternal behavior were affected when pregnant rats were exposed to PCB 77 or when they raised pups that had been exposed to the contaminant during fetal life. Comparing the combined PCB-exposed groups to the oil-only group, we found an increase in the amount of time the dams spent grooming the pups and an increase in the number of nursing bouts as well as in the amount of time the dams spent at the nest, but a reduction in the display of the high-crouch nursing posture. In a recent study, using a conventional design without cross-fostering, we also detected effects of PCB 77 on the maternal behavior of rats, which also included an increase in time spent on the nest, an increase in licking and grooming of the offspring, and a decrease in the display of the high-crouch nursing posture (Simmons *et al.*, 2005). In both studies there were no effects of any type of PCB exposure on the amount of time the dams devoted to nursing the pups. Using mice, experiments with estrogenic endocrine disrupters (Bisphenol A and methoxychlor) administered during gestation without cross-fostering of the litters have also documented changes in maternal behavior (Palanza *et al.*, 2002a and 2002b). In contrast to our results with PCB 77, these estrogenic agents produced a decrease in time on the nest and in time nursing the litters. Despite differences between the specific behavioral effects of particular contaminants in mice and rats, taken together these studies serve to highlight the potential importance of maternal contributions in the evaluation of developmental outcomes in toxicology studies involving gestational

exposure. Interestingly, field studies of avian behavior have reported effects of PCB exposure on nest-site presence and nest building in gulls (Bustnes *et al.*, 2001) and in tree swallows (McCarty and Secord, 1999a and 1999b). Therefore, effects of environmental contaminants on parental behaviors are likely a general feature of contaminant exposure in vertebrates.

Cross-fostering Analysis: Developmental Outcomes

Our results indicate that some developmental outcomes associated with PCB exposure may be independent of any effects of the contaminant on maternal care, while others may be sensitive to the condition of the lactating dam. Thus, the increase in pup mortality appears to be due primarily to exposure of the litters to the contaminant *in utero*, with no substantial protection seen when the PCB-treated pups are reared by oil-treated dams, and with no added morbidity ascribable to being reared by PCB-treated dams. In contrast, evidence for a contribution of postnatal conditions can be seen in the decreased amount of weight gained by pups with gestational and/or lactational exposure to the PCB. Deficits in the display of high-crouch nursing shown by dams treated with PCB 77 or raising PCB-exposed pups likely contributed to the slower growth of the pups, since milk let-down is more likely to occur when the dam is engaged in the high crouch nursing posture than any of the other nursing postures (Lincoln *et al.*, 1980; Voloschin and Tramezzani, 1979; Lonstein *et al.*, 1998).

While some of the effects we see after PCB exposure (e.g., decreased weight gain of the pups) have also been demonstrated after exposure to other teratogens (e.g., lead, mercury, fetal alcohol exposure), it is not likely that these compounds are effecting their change in the same way. For example, PCB-induced AhR gene expression can alter thyroxine levels, which could help to explain the decrease in weight gain we saw after PCB exposure. The potential mechanisms by which lead and ethanol reduce growth in exposed organisms could be much different, and are not likely to work via the AhR since they are not potent ligands for the receptor.

Cross-fostering Analysis: Maternal Behavior

The use of a cross-fostering paradigm allows us to begin to separate effects on maternal behavior that are a result of the PCB actions on the dam from those that are produced in response to a change in the litter brought about by PCB exposure. However, our results show that the contributions of direct versus indirect effects of PCB on maternal behavior are not always clear cut, and that for specific maternal behaviors, both types of effects are likely to be involved.

For maternal autogrooming, our results strongly suggest that the treatment received by the pups *in utero* was responsible for the increase seen in this behavior. Thus, regardless of the treatment that they received during gestation, dams rearing pups that were exposed to PCB *in utero* showed increased autogrooming. PCB exposure *in utero* leads to a decrease in litter size and, therefore, a reduction in the amount of pup-related stimulation

that the dam receives. This reduction in stimulation (e.g. rooting and suckling) may be responsible for the increase in maternal autogrooming as supported by the negative correlation seen here between this behavioral measure and litter size. In addition, direct actions of the PCB on the offspring may have decreased the quality of stimulation provided by the pups that were still present. In order for a dam to engage in the high crouch nursing posture, she must receive an adequate amount of ventral stimulation (Stern, 1996). It is possible that the dam is attempting to compensate for the reduced stimulation provided by the pups by increasing the amount that she licks and grooms her ventrum. In order for this to be the case the increase in autogrooming would be specific to the ventrum of the dams. However, we are not able to distinguish between ventral and dorsal autogrooming and cannot test this hypothesis at this time.

Palanza *et al.* (2002a and b) also observed an increase in autogrooming when mice were treated with estrogenic endocrine disrupters during pregnancy. However, they did not report a reduction in the size of the litters, so PCB 77 and other estrogenic contaminants may produce similar effects on maternal autogrooming, but may act via different mechanisms.

Using a conventional design with no cross-fostering, previous research in this laboratory found an increase in pup grooming when a high dose of PCB 77 (4 mg/kg) was administered during gestation (Simmons *et al.*, 2005). In that study, a 2 mg/kg dose did not affect this measure. Here we report a small facilitation of the behavior by the 2

mg/kg dose that was only detected when the combined data for all PCB groups were compared to the data from the oil-only group.

The PCB-induced alteration seen in pup-directed licking and grooming may be attributable to some of the known biological actions of PCB 77. For example, PCB 77 has been shown to act as an estrogen (Seegal *et al.*, 2005; Nesaretnam *et al.*, 1996), and as such, it may be acting via brain oxytocin systems (Bale and Dorsa, 1995; Pedersen and Boccia, 2003) to increase pup-directed grooming (Champagne *et al.*, 2001; Francis *et al.*, 2000; Champagne *et al.*, 2003). Exposure to other estrogenic contaminants (Palanza *et al.*, 2002a and b) has not resulted in increases in pup-directed licking, but various dioxin-like compounds have increased estradiol secretion in placental tissue in vitro and could, as a consequence, activate OT secretion from the pituitary (Augustowska *et al.*, 2003).

Enhanced licking may also reflect changes in brain dopamine (DA), since changes in levels of brain DA have been shown to affect pup-directed licking and grooming (Champagne *et al.*, 2004; Keer and Stern, 1999; Stern and Taylor, 1991). Developmental exposure to PCBs is known to affect brain DA in the adult (Seegal *et al.*, 1997). If adult exposure to PCBs has similar effects, then behavioral changes in the PCB-exposed dams may stem from effects of the contaminant on brain DA systems. However, there are conflicting data on whether or not PCB exposure to the adult animal causes a similar outcome to perinatal PCB exposure (see Seegal *et al.*, 2005 and Mariussen *et al.*, 1999). Alternatively, changes in the stimulus quality of pups exposed to PCB during gestation and/or lactation may induce more pup-directed grooming from the dams.

The increase in licking of the pups may serve to buffer or compensate for some of the toxic effects that the offspring may otherwise incur as a result of exposure to the contaminant. Manipulations that increase licking and grooming have been shown to protect the offspring from the effects of many early-life insults including fetal ethanol exposure (Lee and Rabe, 1999) and cerebral hypoxia (Chou *et al.*, 2001). It is possible that some developmental effects that would otherwise be seen in the pups as a result of PCB exposure may be lessened because of the increase in licking and grooming that is exhibited by the PCB-exposed groups.

The effect of PCB on the number of nursing bouts was examined in an attempt to understand the effects of the contaminant on pup grooming. Dams engage in more licking and grooming just prior to a nursing bout (Stern, 1989). Thus, an increase in the frequency of nursing bouts induced by actions of PCB 77 could be responsible for the increase in maternal licking. Although a comparison of all PCB 77-exposed groups to the oil-only group revealed an increase in the number of nursing bouts, there was no significant correlation between this measure and the amount of licking and grooming displayed by the dams. Further, the pattern of results for nursing bouts was clearly different from the one obtained for licking and grooming, and very similar to the one seen for autogrooming. Namely, dams rearing pups that had been exposed to PCB 77 during gestation preferentially showed an increase in the display of the behavior. Reduced stimulation by smaller litters may contribute to this behavioral effect as suggested for autogrooming.

Although nursing *per se* was not affected by PCB exposure, dams in the combined PCB groups spent less time in the high crouch nursing posture than their oil-only counterparts. A similar reduction in high crouch was seen previously with the dose given in this experiment as well as with a higher one (4 mg/kg) (Simmons *et al.*, 2005). As was observed for the effects of PCB 77 on licking and grooming of the pups, changes in both the dam and the litter appear to contribute to the reduction in high-crouch nursing. The reduction in high crouch nursing may be due to effects of the contaminant on the physiology of the dams. For example, hypothyroidism has been associated with a reduced ability to lactate in multiple animal models (Ben-David *et al.*, 1966; Swanson and Miller, 1973; Tamasy *et al.*, 1984; Tucker, 1994), and contaminants similar to PCB 77 have been shown to interfere with thyroid functioning (Gorski *et al.*, 1988; Gray *et al.*, 1993; Morse *et al.*, 1993; Seo *et al.*, 1995). But changes in the pups may also contribute to the reduction in high crouch nursing. The litters exposed to PCB either *in utero* or via lactation were lighter in body weight than control litters. Therefore, the reduction in high crouch nursing may result from a reduction in ventral stimulation of the dam by the smaller pups of the PCB-exposed groups. Regardless of questions about mediating mechanisms, the reduction in high crouch nursing induced by exposure to PCB 77 is clearly not indicative of a general neglect for the litter by the dams. In fact, the dams from the combined PCB-exposed groups spent more time on the nest than those from the oil-only group as was reported earlier (Simmons *et al.*, 2005).

Finally, it is important to note that while the dose of PCB used here (2 mg/kg body weight per day) is much larger than an average individual would experience, it is closer

to the amount of PCB exposure received by those individuals living in highly-contaminated areas such as the Arctic. It is important to elucidate the effects of PCBs on organisms so that we can better understand how we may be able to potentially ameliorate the effects of the contaminant after exposure. In order to do this, a higher dose must be used in order to better identify all of the consequences resulting from environmental contaminants.

In summary, our results confirm that exposure to PCB 77 during gestation and/or lactation can have significant effects on the maternal behavior of the dams. The patterns of behavioral changes that are discerned using a cross-fostering paradigm suggest that changes in maternal behavior are likely to emerge from direct effects of PCB on the dams as well as in response to effects of the PCB on the litter. The changes in maternal behavior are not reflective of a global disruption of maternal care since some behaviors that contribute to the survival of the litters are enhanced by the treatment. These and other findings (Simmons *et al.*, 2005; Palanza *et al.*, 2002a and b) should guide and inform future experiments involving gestational treatments that may alter maternal behavior. This is especially important for those studies that examine the effects of contaminants or other perinatal treatments on the behavior of the offspring, since changes in offspring behavior may be rooted in alterations of maternal care brought about by the perinatal treatment as well as any direct treatment effects.

Chapter 4. How does an intermediate dose of PCB 77 affect maternal behavior when using a cross-fostering design?

ABSTRACT

Polychlorinated biphenyls, or PCBs, are environmental toxins that have known behavioral and neurological effects. Research in our laboratory has shown an effect of 3, 4, 3', 4'-tetrachlorobiphenyl (PCB 77) on the maternal care of rats when the PCB was delivered during pregnancy. A dose of 4 mg/kg resulted in altered maternal behavior, but also led to a significant decrease in the number of viable offspring. A subsequent experiment using a cross-fostering design and a dose of 2 mg/kg demonstrated significant effects on maternal behavior with some of these effects resulting primarily from effects of the PCB on the dams and others driven by effects of the PCB on the pups. However, not all of the changes demonstrated after treatment with 4 mg/kg PCB were observed after exposure to 2 mg/kg. The current study, utilizing a dose of 3 mg/kg and a cross-fostering design, was conducted in order to (1) use an intermediate dose of PCB in an attempt to identify whether the changes in maternal care seen after treatment with 4 mg/kg (that were not seen after treatment with 2 mg/kg) were due to PCB-affected pups or dams, and (2) expand the behavioral analysis of the effects of the PCB on the dams to test the "maternal compensation hypothesis." Pregnant rats were treated with subcutaneous injections of either PCB 77 emulsified in corn oil (3 mg/kg b.w.) or vehicle alone on gestation days 6 – 18. Litters were cross-fostered and maternal care was examined on postnatal days 1, 2, 4 and 6. Our data indicate that exposure to PCB 77

altered the amount of time the dams spent on the nest, the time licking and grooming their pups, the time engaged in high crouch nursing, ventral autogrooming, and nursing. These results confirm that exposing dams to PCBs during gestation or to pups that received *in utero* PCB exposure affects maternal behavior in ways likely to contribute to the development of the litter.

INTRODUCTION

Although the use of a cross-fostering design to examine the effects of PCB 77 on maternal behavior answered many of the questions that arose after the Simmons *et al.* study, new questions were also raised by the results. For example, behavioral measures were altered in dams that did not receive PCB treatment directly but reared prenatally PCB-exposed offspring. These dams groomed themselves more and exhibited less high crouch nursing than dams rearing pups exposed to only oil prenatally. In the discussion of those observations, I suggested that the stimulus properties of the PCB-treated pups might have been altered, resulting in the observed change in the dams' behavior. Dams require a specific amount of ventral stimulation from pups in order to achieve the high crouch nursing posture (Stern, 1996). This stimulation usually occurs while the pups are ventrally rooting around for and attaching to a nipple prior to nursing. If the dams do not receive the necessary stimulation, they will not engage in high crouch nursing (Stern and Johnson, 1990; Stern and Lonstein, 1996). I hypothesized that the increase in autogrooming exhibited by dams rearing prenatally PCB-exposed pups was the result of the dams' attempt to compensate for the reduced stimulation provided by these pups.

However, since the original analysis did not separate ventral autogrooming from that directed toward the rest of the body, I was not able to test that hypothesis.

In addition, I was curious as to how maternal behavior was affected by a higher dose of PCB 77 using a cross-fostering design. Even though treatment with the 4 mg/kg dose affected maternal behavior (Simmons *et al.*, 2005), the interpretation of the results was complicated by the relatively high toxicity associated with that dose (as indicated by the appearance of the pups, increase in mortality rate and decrease in the number of dams that delivered litters). Furthermore, there were behaviors that were altered after treatment with 4 mg/kg that were not affected with the 2 mg/kg dose (e.g., pup licking and grooming), and since the experiment using 4 mg/kg did not utilize a cross-fostering design, we were not able to identify if PCB-affected offspring lead to the increase in pup grooming, or if this behavior was changed as a result of the PCB treatment the dam received.

Finally, in a report that examined the effects of various teratogens on development, a 3 mg/kg dose of PCB 77 resulted in morphological effects on the offspring (Wardell *et al.*, 1982). Knowing that treatment with 3 mg/kg causes developmental effects in the exposed offspring, it became important to explore how the same treatment would affect maternal behavior, and how maternal care might be altered in dams responding to prenatally PCB-exposed pups.

For the current experiment, I utilized a cross-fostering design and a more detailed behavioral analysis to examine the effects of 3 mg/kg of PCB 77 on maternal care in rats. Similar to the earlier studies, pregnant females were treated on GD 6 – 18 and litters were cross-fostered on the day of birth. Maternal behavior was analyzed on postnatal days (PNDs) 1, 2, 4 and 6, and developmental outcomes of the offspring were studied on PND 6.

METHODS

Animals and Housing

Twenty-four timed-pregnant Long-Evans rats from Charles River Laboratories (Raleigh, North Carolina) arrived at our laboratory on gestation day (GD) 5, and were maintained on a 14:10-h light:dark cycle with lights off at 1100 h at an ambient temperature of 21°C in a laminar-flow unit (NUAIRE). A dim red light remained on at all times. The rats were housed singly in plastic cages (45 X 22 X 21 cm) with wood shavings as bedding, and were allowed free access to water and Harlan Rodent Chow 8640 food pellets. Animals were maintained in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, and the Michigan State University Animal Care and Use Committee approved experimental procedures.

Procedure

The dams were weighed daily and given subcutaneous injections of the oil vehicle or 3, 4, 3', 4'-tetrachlorobiphenyl (PCB 77; Accustandard, New Haven, CT; >99% pure as determined by gas chromatography performed by the vendor) emulsified in corn oil in the dose of 3 mg/kg from GD 6 to GD 18. Nest building materials in the form of thirty brown paper towel strips were placed on the wire top of each cage on GD 20. Parturition took place during the mid- to late-light phase of the animals' light/dark cycle on GD 22 - 23. On the day of birth [day of birth = postnatal day (PND) 0], the litters were culled to 8 animals (4 females and 4 males) and cross-fostered or left with their own mothers to create the following groups: (1) oil treated dams with their own oil pups, (2) PCB treated dams with their own PCB pups, (3) oil treated dams with a litter of PCB-exposed pups, and (4) PCB treated dams with a litter of oil pups. Each group contained six dams.

Dams and litters were videotaped on PND 1, 2, 4 and 6 for the animals' last hour of light and first hour of dark (2 hr/day/animal). Videos were analyzed using The Observer 4.1 (Noldus Information Technology), a behavioral data acquisition computer program. During review of the videotapes maternal behaviors were recorded as was described in detail in Cummings *et al.*, 2005. Briefly, the behaviors that were analyzed include the amount of time the dam spent on the nest, licking and grooming the pups, autogrooming ventrally and dorsally, and amount of time spent nursing. Nursing behaviors included resting, low crouch and high crouch. Time spent nursing is the total of all time spent in one of the three nursing postures while on the nest. The number of nursing bouts, defined

as visits to the nest that involved nursing of the litter, was also recorded. Average pup weight gain and pup survival between PND 1 and PND 6 were determined for all litters. All percentages are relative to the two-hour scoring period, except for the percentages for the nursing behaviors, which were calculated from the total time spent nursing.

Two investigators scored the maternal behaviors from the video recordings. Because of procedural demands, it was impossible to keep the investigators unaware of the treatment received by each dam. A subset of the recordings was scored independently by both investigators and a correlational analysis was used to evaluate the inter-observer reliability. For all the maternal behaviors analyzed, the positive correlation between the two data sets was greater than .90.

Analyses

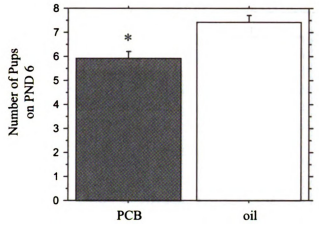
For each behavioral measure, a 2 X 4 ANOVA (treatment groups X days, repeated measures on the second factor) was used to test the hypothesis that exposure to PCB by the dam and/or the litter affects the dams' behavior. This was evaluated by comparing the oil group to a combined PCB group that included all groups receiving any PCB exposure. The cross-fostering design included four treatment groups and 4 X 4 ANOVAs (treatment groups X days) with repeated measures on the second factor were used to analyze the data for significant main effects of treatment and day and the interactions. Significant overall *F* ratios were followed by analysis of the simple effects and/or post hoc comparisons of the individual group means using the Fisher's PLSD test. The data

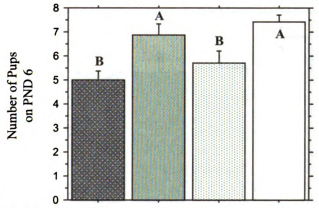
on pup survival and weight gain of the litters were analyzed using *t*-tests (oil vs. combined PCB groups) and one-way ANOVAs (comparing all four treatment groups), followed by appropriate post hoc comparisons. All differences considered statistically significant have $p \leq 0.05$.

RESULTS

A comparison of all PCB-exposed groups to the oil-only group showed that PCB decreased the number of pups present on PND 6 ($t = -2.84$, df = 25, $p = 0.009$; Figure 10, Top). The ANOVA analyzing the effect of the four treatment groups on pup survival found a significant main effect of treatment [$F(3, 23) = 6.97$, $p = 0.002$], with post hoc tests indicating that pups who received prenatal PCB exposure had a higher mortality rate than pups who received prenatal oil exposure (Figure 10, Bottom).

The *t*-test comparing the combined PCB groups to the oil-only group detected a significant effect of PCB on the average amount of weight gained by the pups between PNDs 1 and 6 ($t = -3.27$, df = 25, $p = 0.003$; Figure 11, Top). The analysis of the four separate treatment groups identified a significant main effect of treatment on pup weight gain [$F(2, 23) = 3.36$; $p = 0.036$], with post hoc analysis indicating that any exposure to PCB decreased the average amount of weight gained by the pups over the first six postnatal days compared to the oil only group (Figure 11, Bottom).





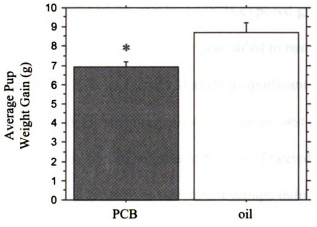
Prenatal pup Treatment

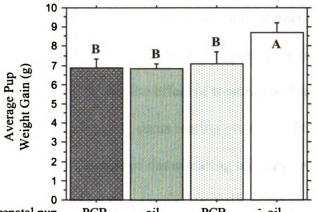
(Treatment of biological dam)

Postnatal pup Treatment	PCB	PCB	oil	oil

(Treatment of dam rearing litter)

Figure 10 Top: A comparison of the oil-only group against the combined PCB group found a significant difference (*) in the number of pups present on PND 6, with fewer pups surviving in the combined PCB group. Bottom: A comparison of the four treatment groups using a one-way ANOVA revealed a main effect of treatment on pup survival; the groups sharing the same letter are not significantly different from each other.



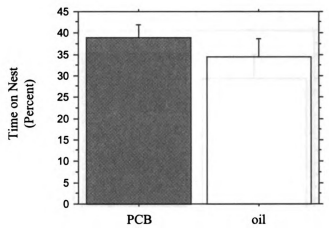


Prenatal pup Treatment	PCB	oil	PCB	oil
(Treatment of biological dam)				
Postnatal pup Treatment	PCB	PCB	oil	oil
(Treatment of dam rearing litter)				

Figure 11 Top: A comparison of the oil-only group against the combined PCB group found a significant difference (*) in the average amount of weight that the pups gained from PND 0 to PND 6, with less weight gained by the combined PCB group. Bottom: The ANOVA of the data from the four treatment groups revealed a significant main effect of treatment; groups with the same letter are not significantly different from each other.

The two-way ANOVA comparing the combined PCB-exposed groups to the oil only group for percent of time the dams spent on the nest failed to reach significance for effect of PCB ($p = 0.548$; Figure 12, Top), but did identify a significant effect of days [$F(1, 25) = 2.73, p = 0.050$] with the dams spending more time on the nest on PND 1 than on PNDs 4 and 6 (data not shown). No significant interaction was detected for this measure ($p = 0.586$). The ANOVA of the four different treatment groups indicated that both days [$F(3, 96) = 5.17, p = 0.003$] and treatment [$F(3, 23) = 3.69, p = 0.026$] had a significant effect on percent of time on the nest, with no significant interaction ($p = 0.401$). For the effect of days, post hoc analysis revealed that dams spent more time on the nest on PND 1 as compared to PNDs 4 and 6. For the effect of treatments (Figure 12, Bottom), post hoc analysis showed that PCB-treated dams rearing prenatally PCB-exposed pups spent more time on the nest than PCB-treated dams rearing oil pups. Also, oil-treated dams rearing pups that had received PCB exposure prenatally spent more time on the nest than dams rearing pups that had been exposed to oil prenatally (Figure 12, Bottom).

For percent of time spent engaged in pup-directed LG, the ANOVA for the combined PCB groups and the oil-only group failed to identify significant effects of PCB ($p = 0.463$; Figure 13, Top), of days ($p = 0.923$) or an interaction ($p = 0.270$). The analysis of the four treatment groups detected a significant interaction between treatment and PND [$F(9, 69) = 2.09, p = 0.042$]. There was a simple main effect of PND [$F(3, 24) = 3.21, p = 0.041$] for oil dams rearing prenatally PCB-exposed pups, with dams spending more time licking the offspring on PNDs 1 and 2 than PND 4 (data not shown). There was a significant simple main effect of treatment on LG on PND 1 [$F(3, 23) = 2.97, p = 0.050$]



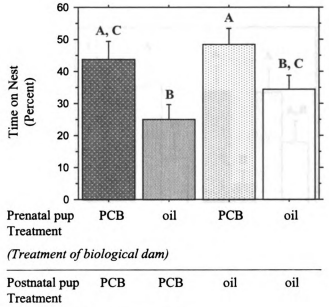


Figure 12 Top: A comparison of the oil-only group against the combined PCB group did not find a significant difference in the amount of time the dam spent on the nest ($p = 0.55$). Bottom: The ANOVA of the data from the four treatment groups found a significant main effect of treatment on time spent on the nest; the groups sharing the same letter are not significantly different from each other.

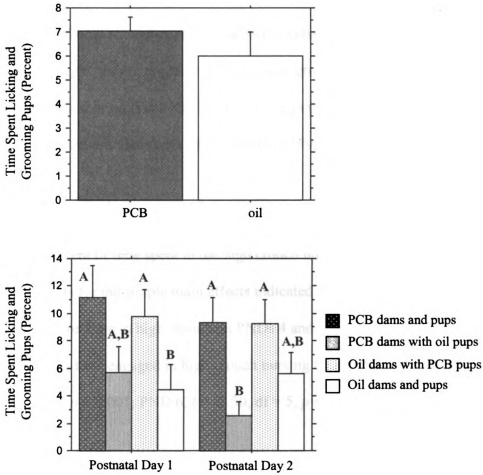
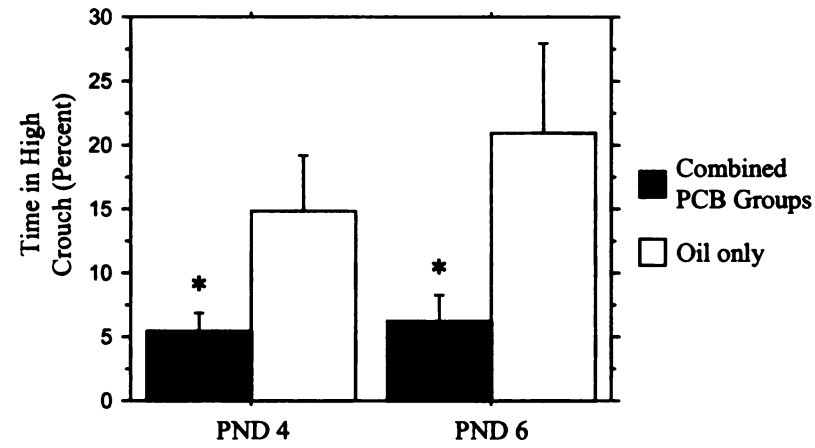


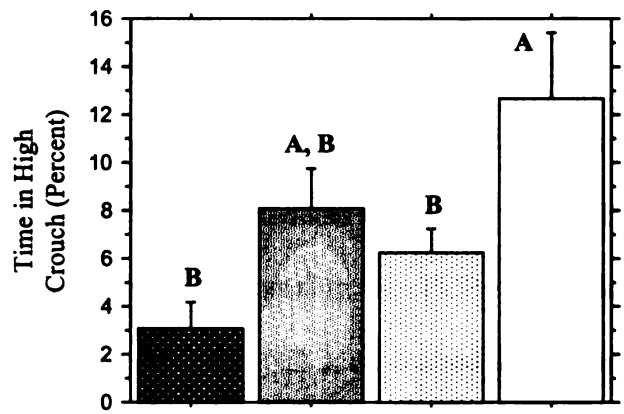
Figure 13 Top: A comparison of the oil-only group against the combined PCB group did not detect a significant difference in the amount of pup-directed licking and grooming (LG; $p = 0.46$). Bottom: The ANOVA of the data from the four treatment groups revealed a significant interaction between treatment and postnatal day for LG; groups sharing the same letter are not significantly different from each other.

and PND 2 [F (3, 23) = 4.36, p = 0.014], but not on PND 4 (p = 0.453) and PND 6 (p = 0.982) (data not shown). Dams rearing pups that had been prenatally exposed to PCB spent more time grooming the pups compared to the oil only group on PND 1 (Figure 13, Bottom). On PND 2, dams rearing prenatally PCB-exposed pups spent more time engaged in LG than PCB-treated dams with oil-treated pups (Figure 13, Bottom).

A significant interaction was detected by the ANOVA of the combined PCB groups and the oil only group for percent of time spent in the high crouch nursing posture [F (3, 75) = 3.22, p = 0.028]. T-tests for the simple main effects indicated that there was a significant effect of PCB on time in high crouch on PNDs 4 and 6, with the combined PCB groups spending less time engaged in high crouch nursing than the oil only group (PND 4, t = -9.02, df = 5, p < 0.001; PND 6, t = -2.50, df = 5, p = 0.050; Figure 14, Top).

There was an effect of days for the oil only group [F (3, 4) = 14.19, p = 0.013] with dams spending more time in the high crouch posture on PND 6 compared to PNDs 1, 2 and 4, and more time on PND 4 than on PND 1. There was no effect of days for the combined PCB groups. There was no effect of PCB exposure on high crouch nursing on PNDs 1 (p = 0.403) and 2 (p = 0.350) (data not shown). The ANOVA of the four treatment groups also detected a significant effect of treatment on this measure [F (3, 23) = 3.82, p = 0.020], but no effect of PND (p = 0.284) and no significant interaction (p = 0.381). Post hoc analysis indicated that dams rearing pups that had been exposed to PCB prenatally spent less time engaged in high crouch nursing than the oil-only control group (Figure 14, Bottom).





Prenatal pup Treatment	PCB	oil	PCB	oil
(Treatment of biological dam)				
Postnatal pup Treatment	PCB	PCB	oil	oil
(Treatment of dam rearing litter)				

Figure 14 Top: A comparison of the oil-only group against the combined PCB group found a significant interaction between treatment and day for the amount of time the dam spent in high crouch. Analysis of the simple main effects revealed a significant difference (*) in the display of high crouch nursing on PNDs 4 and 6, with the PCB group exhibiting less high crouch nursing. Bottom: The ANOVA of the data from the four treatment groups found a significant main effect of treatment on the display of high-crouch nursing; the groups sharing the same letter are not significantly different from each other.

A significant increase in maternal ventral autogrooming as a result of PCB exposure was detected when comparing the combined PCB groups to the oil only group [F (1, 25) = 4.40, p = 0.046] (Figure 15, Top) with no effect of days (p = 0.067) and no interaction (p = 0.395). When the four separate treatment groups were compared, the ANOVA detected a significant interaction between treatment and days [F (9, 69) = 4.34, p < 0.001]. Analysis of the simple main effects found a significant effect of treatment on PND 1 [F (2, 23) = 11.20, p < 0.001] and PND 2 [F (3, 22) = 4.59, p = 0.012; Figure 15, Bottom] but not PND 4 (p = 0.968) and PND 6 (p = 0.772) (data not shown). Post hoc analysis found that on PND 1, PCB-treated dams rearing prenatally PCB-exposed pups spent more time grooming themselves ventrally than the other three treatment groups (Figure 15, Bottom). On PND 2, PCB-treated dams with prenatally PCB-exposed pups spent more time engaged in ventral autogrooming than PCB-treated dams rearing oil-treated pups (Figure 15, Bottom). Also on PND 2, oil-treated dams rearing prenatally PCB-exposed pups groomed themselves ventrally more than dams rearing prenatally oil-treated pups (Figure 15, Bottom). There was a significant simple main effect of days for PCB dams rearing PCB pups [F (3, 20) = 12.77, p < 0.001] but not for the other three groups. PCB dams rearing PCB pups spent more time engaged in ventral autogrooming on PND 1 than on the other three PNDs. The amount of time spent engaged in dorsal autogrooming was not affected by PCB exposure (p = 0.278; data not shown).

The one-way ANOVA did not detect a significant main effect of treatment or of days on the percent of time spent nursing when comparing the combined PCB groups to the oil-only control (Figure 16, Top). However, the ANOVA of the four treatment groups

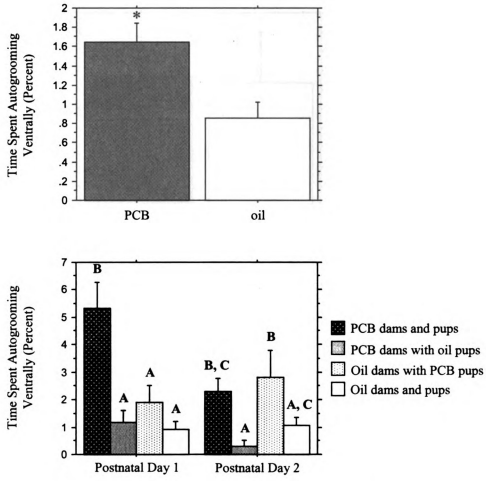
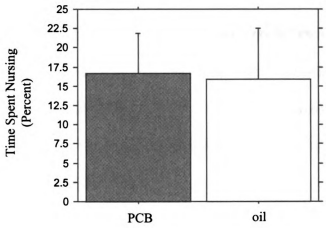
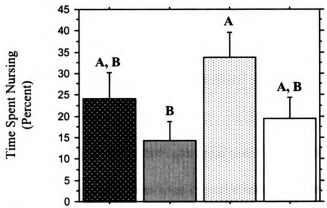


Figure 15 Top: A comparison of the oil-only group against the combined PCB group found a significant difference (*) in the amount of time the dam engaged in ventral maternal autogrooming, with an increase in ventral autogrooming seen in the combined PCB group. Bottom: The ANOVA of the data from the four treatment groups found a significant interaction between treatment and day for time spent autogrooming ventrally; the groups sharing the same letter are not significantly different from each other.





Prenatal pup Treatment	PCB	oil	PCB	oil
(Treatment of biological dam)				
Postnatal pup Treatment	PCB	PCB	oil	oil
(Treatment of dam rearing litter)				

Figure 16 Top: The difference between percent of time spent nursing for the oil-only group and the combined PCB group missed statistical significance ($p = 0.79$). Bottom: The ANOVA of the data from the four treatment groups found a significant main effect of treatment on time spent nursing; the groups sharing the same letter are not significantly different from each other.

identified a significant main effect of treatment [F (3, 23) = 2.92, p = 0.050] and of days [F (3, 69) = 2.80, p = 0.046], with no significant interaction between the two (p = 0.695). Post hoc analysis indicated that oil-treated dams rearing prenatally PCB-exposed pups spent more time nursing than PCB-treated dams rearing oil-exposed pups (Figure 16, Bottom). Additionally, all dams spent more time nursing on PND 1 than on the other three PNDs (data not shown).

DISCUSSION

Cross-fostering Analysis: Developmental Outcomes

The effects of 3 mg/kg PCB on the developmental outcomes of the offspring paralleled, for the most part, those reported in previous experiments that used a lower dose (2 mg/kg; Cummings *et al.*, 2005) and a higher dose (4 mg/kg; Simmons *et al.*, 2005) of PCB on the same gestation days. Perinatal PCB exposure decreased the number of pups that remained in the nest on postnatal day (PND) 6 when the pups received PCB exposure *in utero*, similar to what was found with the 2 mg/kg dose when using the cross-fostering design (Cummings *et al.*, 2005). The 4 mg/kg dose also increased pup mortality using a traditional treatment paradigm (Simmons *et al.*, 2005). The increase in mortality seen in the prenatally PCB-exposed pups is most likely attributable to the toxic effects of the PCB on the litter in utero. However, it cannot be ruled out that the dam's behavior is changed in response to an alteration in the physiology and/or behavior of the pups, which in turn affects their viability.

PCB exposure with the 3 mg/kg dose also decreased the average amount of weight gained by all offspring that were exposed to PCB regardless of the timing of exposure. That is, pups receiving pre- and/or postnatal PCB exposure gained less weight than offspring who were not exposed to PCB. Treatment with 2 mg/kg in Cummings *et al.* (2005) also decreased pup weight in the combined PCB groups compared to the oil only group, and a similar pattern was found when comparing the four treatment groups to one another. Traditional treatment with 2 and 4 mg/kg (Simmons *et al.*, 2005) also decreased average pup weight gain in pups exposed pre- and postnatally. The reduction in weight gain seen with the 3 mg/kg dose may be due, in part, to the finding that dams in the combined PCB group spent less time in the high crouch nursing posture than oil-treated dams on PNDs 4 and 6. Since the high crouch nursing posture is the one in which the majority of milk letdown occurs (Lincoln *et al.*, 1980; Voloschin and Tramezzani, 1979; Lonstein et al., 1998), it is reasonable to suggest that pups who receive less high crouch nursing would not gain as much weight as those pups whose mothers were engaged in the nursing posture a higher percentage of the time.

Cross-fostering Analysis: Maternal Behavior

For the 3 mg/kg dose of PCB used here, the analysis comparing the four treatment groups to one another revealed significant treatment effects on various aspects of maternal care. Interestingly, the majority of the affected behaviors tended to be changed as a result of dams rearing prenatally PCB-exposed pups.

The amount of time dams spent licking and grooming their pups on PNDs 1 and 2 was altered in dams rearing pups that were exposed to PCB prenatally compared to dams that reared prenatally oil-exposed pups. Treatment with 4 mg/kg PCB using a conventional design with no cross-fostering also increased LG (Simmons *et al.*, 2005) and, while treatment with the lower dose of PCB (2 mg/kg; Cummings *et al.*, 2005) did not result in significant differences between the four groups for this measure, it did lead to an increase in pup-directed LG when comparing the combined PCB groups to the oil-only group.

After analyzing the effects of 2 mg/kg PCB on maternal behavior in the previous experiment (Cummings *et al.*, 2005), I hypothesized that the increase in pup-directed LG may have been due, in part, to the PCB acting as an estrogen via brain oxytocin systems to increase the amount of LG displayed by the dams. Additionally, I suggested that PCB-induced alterations in brain dopamine (DA) could play a role in the increase seen in LG, since developmental exposure to PCB has been shown to increase brain DA in adults and alteration in brain DA have been shown to affect pup-directed LG. Support for these hypotheses requires that the increase in LG be shown only by those dams that received PCB treatment during pregnancy, but not by those dams that received oil treatment and reared PCB-exposed offspring, as was the case here. Therefore, the increase in pup-directed LG appears to be due not to the direct effects of the toxin on the dams, as suggested previously, but to a change in the prenatally PCB-exposed pups that in turn affects the behavior of the dams.

One way in which pups could have been changed is through PCB-induced masculinization. Moore and Morelli (1979) have shown that male pups receive more pup-directed LG from the dam than their female siblings. Treatment of neonatal female pups with testosterone or estrogen increased the amount of LG that the female pups received so that they no longer differed from males (Moore, 1982). Since PCB can act as an estrogen (Jansen *et al.*, 1993; Nesaretnam *et al.*, 1996) and pups that have been exposed to PCB prenatally receive more LG than oil pups, it is possible that prenatal PCB is acting to masculinize the female offspring, resulting in an increase in the amount of LG provided by the dams. Thus, maternal behavior may have been altered in response to a change in the stimulus properties of the pups, particularly in the female offspring.

In addition to masculinization, PCB may have affected how the pups interact with their mother. For example, dams often groom their pups at the start of a nesting bout prior to nursing (Stern, 1989). During this time, the dam receives ventral stimulation from the pups while the pups are rooting around for and attaching to a nipple. After the dam receives sufficient stimulation she will become immobile and display the high crouch nursing posture. If she does not receive the ventral stimulation required to assume the posture, because of a change in the behavior of the pups or a decrease in the number of pups present, the dam will not adopt the nursing posture (Stern and Johnson, 1990; Stern and Lonstein, 1996). Leigh and Hofer (1973) have shown that when the number of pups in the litter is reduced to one, the dam will continue to lick that pup longer than she would have had she received enough stimulation from the offspring to stop licking and to display the high crouch nursing posture. Pups that were exposed to PCB prenatally

weigh less and are present in fewer numbers on PND 6 than oil only controls, and the dams that rear these pups spend more time licking their pups and less time engaged in high crouch nursing than dams with prenatally oil-exposed pups. A litter that is smaller in size and number may not provide enough ventral stimulation to stop the dam's display of pup-directed LG and elicit the high crouch nursing posture, leading to an overall increase in LG and decrease in high crouch.

The interaction that occurred between treatments and days for high crouch nursing may be related to the decrease in average amount of weight gained by PCB-exposed pups. The display of high crouch is reduced on PNDs 4 and 6 in the combined PCB groups. The average amount of weight gained by the offspring in these groups is also reduced compared to the oil-only controls. Previous research has shown that high crouch nursing is more prevalent in later postpartum compared to early postpartum (Stern and Johnson, 1989); the offspring are larger and can more easily elicit the high crouch behavior from the dam. Since a dam will only engage in high crouch nursing if she receives adequate ventral stimulation, pups that are smaller in size may be less able than larger pups to stimulate the dam to display the high crouch posture. The difference in high crouch nursing may not develop between treatment groups until PND 4 because the groups may not exhibit a difference in weight until that time.

Since this experiment did not look at average pup weight gain on each PND, it is not possible to know if the difference in weight gain emerged just after birth or later in the first postnatal week. However, a previous experiment using 2 and 4 mg/kg doses of PCB

did not find a difference in pup weight gain until PND 2 (Simmons *et al.*, 2005). This finding may lend support to the suggestion that the interaction between treatment and days is related to changes in the size of the offspring that are not present immediately after birth. In addition, high crouch nursing is the posture from which the pups receive the most nourishment. Thus, a reduction in high crouch may result in lower weight gain by the young, causing in a circular pattern of cause and effect that would result in decreased pup weight gain and decreased display of high crouch.

PCB exposure altered the pattern of effect of days for high crouch nursing, such that the amount of time the dams in the combined PCB group spent engaged in high crouch did not increase on PNDs 4 and 6, as it did for the oil only group. If the hypothesis presented above is correct and PCB-exposed pups gain weight more slowly than oil-only pups, the amount of high crouch nursing exhibited by the dams in the combined PCB groups may increase at a later date when the size of PCB-exposed pups is sufficient to evoke the behavior. On the other hand, high crouch nursing in the combined PCB groups may not increase to the level of that exhibited by the oil-only females for the duration of lactation. I am unable to address this question, as maternal behavior was not observed beyond PND 6.

Although the 2 and 4 mg/kg doses did not change the pattern of effects of days for any measure, it is not unprecedented for an environmental contaminant to do so (Palanza *et al.*, 2002b). After treatment with the endocrine disrupter methoxychlor (MXC), Palanza *et al.* report an alteration in the regulation of timing of maternal behavior in mice.

Control mice exhibit a natural decline in nest-related behaviors near the end of the second postnatal week, an index of the start of the weaning process. Dams treated with MXC began to decrease the amount of time they spent engaged in nest-related behaviors at the end of the first postnatal week during the dark period. Although the total amount of maternal care that the pups received did not differ, altering the time weaning starts can affect the neuroendocrine and/or behavioral profile (Fleming *et al.*, 1999). Therefore, that PCB treatment altered the effect of days in this study may have significant implications for the offspring.

In an attempt to expand on the behavioral analysis of the previous cross-fostering report (Cummings *et al.*, 2005) the current study analyzed maternal ventral autogrooming separately from all other autogrooming displayed by the dam. If PCB exposure does change the stimulus properties of the pups in addition to reducing them in number, and the dam is unable to achieve and sustain the high crouch, I hypothesized that the increase in autogrooming exhibited by dams rearing PCB-exposed pups may reflect an effort by the dam to compensate for reduced stimulation from the pups through increased ventral autogrooming. The present data confirm that on PND 2, dams rearing prenatally PCB-exposed pups spent more time engaged in ventral autogrooming than dams rearing oil-exposed pups. This finding lends support to the hypothesis that the increase in maternal autogrooming displayed by dams rearing PCB pups may be the result of an attempt to self-stimulate because of a decrease in pup-provided ventral stimulation.

The data indicate that the amount of time the dam spent on the nest was also related to the treatment that the pups received gestationally. That is, dams rearing pups that had been exposed to PCB prenatally tended to spend more time on the nest than dams rearing prenatally oil-exposed pups. This result is similar to what was reported after treatment with 2 and 4 mg/kg. The increase in this behavior may be partly a result of the increase in amount of time these dams spent grooming both themselves and their offspring on PNDs 1 and 2. Oil-treated dams rearing prenatally PCB-exposed pups tended to spend more time nursing than the other groups, which may also help to account for the increased amount of time spent on the nest.

Finally, the amount of time spent nursing was altered after treatment with 3 mg/kg PCB. This is the first time PCB 77 has been shown to affect this measure, although time spent nursing has been modified before with exposure to the environmental contaminants methoxychlor (Palanza *et al.*, 2002b) and Bisphenol A (Palanza *et al.*, 2002a), which are also capable of estrogenic activity. Although PCB dams with oil pups and oil dams with PCB pups differed from one another, they did not differ significantly from the oil-only group or the group of PCB-treated dams rearing prenatally PCB-exposed pups. This pattern of behavior has not been exhibited in any other of the behaviors analyzed, including the specific nursing postures that make up the total time nursing. Thus, while oil dams rearing PCB pups did not spend significantly more time in any specific nursing posture (and even spent less time in high crouch than the oil only group) the mean amount of time spent in each position must have been elevated enough to put their total

time nursing above that for PCB dams rearing oil offspring. The mechanism for this, however, is unknown.

It is unclear as to why this dose (3 mg/kg PCB 77) resulted in interactions between treatments and days and the 2 and 4 mg/kg doses did not. The lower dose may not have had enough impact on the animal in order to affect the pattern of behavior over days, whereas the main effect of the higher dose of PCB may have been so salient as to obscure any potential interactions. It is possible that 3 mg/kg may be a threshold dose that is strong enough to evoke interactions between treatment and postnatal days, but not so strong as to cancel out the interactions because of its strong main effects.

Summary

Our results confirm that treatment with 3 mg/kg PCB 77 alters maternal care and offspring developmental measures. The patterns of behavioral change indicate that these alterations in maternal care were most often due to the dams' response to prenatally PCB-exposed pups as opposed to being direct effects of PCB treatment on the dam. This treatment yielded results that parallel those produced with a 2 mg/kg treatment. Both doses caused a decrease in pup weight gain, number of pups on PND 6, and time spent engaged in high crouch. A similar pattern resulted for percent of time PCB-exposed animals spent on the nest and licking and grooming the pups, but only treatment with the lower dose caused these behaviors to change significantly. The amount of time the dams spent nursing was not significantly different after either dose.

It is clear that evaluation of the effects of environmental contaminants requires extensive analysis to separate out those effects specific to the mother, those specific to the offspring and those that arise as an interaction between a mother and her young.

Chapter 5. Does the sexual behavior or partner preference of pre- and/or postnatally exposed PCB pups differ from controls? If so, are these differences related to the altered maternal care exhibited by the effected dams?

ABSTRACT

Polychlorinated biphenyls (PCBs) are environmental contaminants that cause developmental and behavioral problems in humans and animals. In rats, gestational exposure to the PCB congener 3, 4, 3', 4'- tetrachlorobiphenyl (PCB 77) affects the brain and behavior of the offspring as well as the maternal behavior of the dams. In the present study, a cross-fostering design was utilized to examine the effects of pre- and/or postnatal exposure to PCB 77 on sexual behavior and partner preference in rats. Pregnant rats were injected subcutaneously with either oil or PCB emulsified in oil (2 mg/kg b.w.) on gestation days 6 – 18 and then given pups that had been exposed to either the oil vehicle or PCB during gestation. The offspring were tested as adults in a five-week paradigm that included an initial partner preference test, three sexual behavior tests, and a final partner preference test. A 3-compartment chamber was used for partner preference tests to examine the amount of time each experimental animal spent with the stimulus male and female while allowing for sexual interaction to take place. Our results indicate that PCB 77 affects male and female partner preference but not the display of sexual behavior. Males exposed to PCB both pre- and postnatally showed a significant increase in their preference for the female following the three tests for sexual experience; this effect of sexual experience was absent in the other groups. A comparison of the

combined PCB groups to the oil-only group indicates that PCB increased the amount of time females spent in the vicinity of the stimulus female and decreased the amount of time spent with the stimulus male compared to controls. A comparison of the three different groups of PCB exposure and the oil control showed that females that received postnatal PCB exposure spent more time in the stimulus female chamber than females that received no PCB exposure, and females receiving both pre- and postnatal PCB exposure spent less time with the stimulus male than females receiving either no PCB exposure or prenatal exposure only. The effects of PCB 77 on female partner preference may stem from direct effects of the contaminant on the developing animal, but could be also the result of the enhanced amount of maternal licking and grooming received by these animals during the neonatal period.

INTRODUCTION

As previous experiments have shown (Simmons *et al.*, 2005; this dissertation), rats that receive PCB exposure during pregnancy and/or rats that rear prenatally PCB-exposed offspring exhibit altered maternal behavior. The importance of maternal care in several aspects of offspring development is well documented (see Dissertation Introduction for review). For example, significantly decreasing the amount of time a dam licks and grooms her pups affects the timing of male sexual behavior (Moore, 1984), decreases the number of motor neurons in the sexually dimorphic spinal nucleus of the bulbocavernosus (Moore *et al.*, 1992) and significantly reduces total dendritic arbor in the SNB (Lenz and Sengelaub, 2005). On the other hand, increasing the amount of licking

and grooming (LG) the offspring receive decreases their level of fearfulness when confronted with a novel situation (Caldji *et al.*, 1998) or in response to stress (Liu *et al.*, 97). Pup-directed LG has also been implicated in female reproductive success. Gomes *et al.* (1999) demonstrated that females receiving more LG as a result of neonatal handling showed an increase in the number of anovulatory cycles compared to non-handled females. Thus, alterations in maternal care as a result of PCB exposure have the potential to greatly affect the reproductive development of the offspring.

Previous work in this laboratory has indicated that PCB treatment alters female receptivity and sexual behavior when administered neonatally (Chung *et al.*, 2001) and perinatally (Chung and Clemens, 1999). However, due to the designs of the experiments that examined the effects of PCB on offspring behavior, it is not known whether the effects seen in the offspring are attributable to the direct effects of pre- and or postnatal PCB exposure, or to PCB-induced changes in maternal behavior.

Further, *in vivo* and *in vitro* studies have shown that PCB 77 can have both estrogenic and anti-estrogenic activity (Nesaretnam *et al.*, 1996; Jansen *et al.*, 1993). Estrogen (E) has been shown to be necessary for the development of males' preference for females (Brand *et al.*, 1991; Bakker *et al.*, 1996; Houtsmuller *et al.*, 1994), and the estrogenic metabolite of testosterone is also required for the development of male sexual behavior (see Baum, 2003 for review). Moreover, females given exogenous E early in development demonstrate masculinized sexual behavior when tested as adults (Paup *et*

al., 1972; Paup *et al.*, 1974). Consequently, PCB 77 may be acting estrogenically or anti-estrogenically to alter male and female partner preference and sex behavior.

For the current experiment, I treated pregnant rats with PCB 77 during gestation and tested their offspring as adults to evaluate the effects of prenatal exposure (PCB received while the offspring were in utero) and/or postnatal exposure (PCB received via lactation) on partner preference and sexual behavior. In the analysis, I also considered that the effects seen in the offspring might be due, in part, to PCB-induced alterations in maternal care.

METHODS

Animals and Housing

The animals used in this study were born to the Long-Evans dams from Chapter 2 that received subcutaneous injections of 2 mg/kg PCB 77 on GD 6 – 18. Parturition took place during the mid- to late-light phase of the animals' light/dark cycle on GD 22 - 23. On the day of birth [day of birth = postnatal day (PND) 0], the litters were culled to 8 animals (4 females and 4 males) and cross-fostered or left with their own mothers to create the following groups: (1) oil treated dams with their own oil pups, (2) oil treated dams with a new litter of oil pups, (3) PCB treated dams with their own PCB pups, (4) PCB treated dams with a different litter of PCB-exposed pups, (5) PCB treated dams with a litter of oil pups, and (6) oil treated dams with a litter of PCB-exposed pups. Each

group contained six dams. Two males and two females were weaned from each dam on PND 23 and housed in same-sex pairs for the duration of the study in plastic cages similar to those previously described. Animals were maintained in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, and the Michigan State University Animal Care and Use Committee approved all experimental procedures.

Hormone Treatments

At 60 days of age, two female offspring from each litter were ovariectomized (anesthetized with a ketamine/xylazine cocktail containing 44 mg/kg ketamine, 10 mg/kg xylazine) and implanted subcutaneously with an estradiol benzoate-filled silastic capsule. Capsules were made from silastic brand laboratory tubing (Dow Corning, cat. No. 508-006) with an inner and outer diameter of 0.058 in. and 0.077 in., respectively. Estradiol benzoate (EB, Sigma) was dissolved in ethanol and allowed to sit in a Petri dish for 48 hr or until all of the liquid evaporated from the dish. The crystalline EB was then packed in the pre-cut silastic tubing to fill a length of 5 mm. The ends of the capsules were sealed with medical-grade silastic adhesive (Dow Corning), and implants were incubated in PBS for 24 hr prior to implantation. After one week of recovery and four hours prior to the start of the sexual behavior tests, females were injected with 0.5 mg/0.1 ml progesterone. Stimulus females were at least 60 days of age at the start of testing. They were not ovariectomized but were treated with estrogen and progesterone in a manner similar to experimental females.

Testing Procedure

Males and females began their 5-week testing paradigm when they reached 90 and 60 days of age, respectively. The first week consisted of a partner preference test during which animals were sexually naïve, followed by sexual behavior tests once a week for three weeks, with behavioral data collected only on the third and final test. The paradigm ended with a partner preference test during the fifth week. All tests took place under dim red illumination during the the dark phase of the light-dark cycle.

Partner Preference Tests

Animals were tested in a three-compartment apparatus made of Plexiglas. The middle compartment was connected to the two outer compartments (30 X 59 X 39 cm each chamber, 90 X 59 X 39 cm total) by openings in the back of the apparatus. One of the outer compartments contained a sexually active male and the other a sexually receptive, hormone-treated female. Both stimulus animals were tethered to the front of the apparatus using a Velcro harness and wire. Stimulus animals were able to display sexual behavior but were not able to leave their respective chambers. Experimental animals were able to traverse the chambers freely through the openings in the back of the apparatus. The experimental animal was placed in the center chamber and allowed to acclimate for 5 min prior to the start of the test. After 5 min, the clear plastic divider that closes the middle compartment off from the rest of the apparatus was removed. The test

lasted for 20 min, during which time the duration spent in each chamber was recorded. No sexual behavior was recorded at this time.

Three investigators scored the female partner preference tests from the video recordings. Because of procedural demands, it was impossible to keep one of these three investigators unaware of the treatment received by each offspring. A subset of the recordings was scored independently by all investigators and a correlational analysis was used to evaluate the inter-observer reliability. The positive correlation between the three data sets was greater than .80 for all behavioral measures.

Male-Paced Sexual Behavior Tests

Sexual behavior tests were conducted in a Plexiglas chamber, 56 X 44 X 49 cm, where the experimental male had free access to a sexually receptive stimulus female who received EB implants and progesterone injections as described above. Males were allowed 5 min to acclimate to the chamber before the introduction of the female. The test was terminated after the male achieved a post-ejaculatory intromission or 30 min had elapsed since the female was introduced and no ejaculation was achieved. Behavioral measures that were recorded include: number of mounts and intromissions, mount latency (ML, the time until the male achieved his first mount), intromission latency (IL, the time until the male achieved his first intromission), ejaculation latency (EL, the interval from the first intromission to ejaculation), copulatory efficiency (CE, number of intromissions divided by the number of intromissions plus the number of mounts), inter-intromission

interval (III, the average amount of time between intromissions starting with the first intromission and ending with ejaculation), and post-ejaculatory interval (PEI, the interval from ejaculation to the next intromission).

Female-Paced Sexual Behavior Tests

Experimental females were tested with sexually active adult stimulus males in a two-compartment Plexiglas cage. The testing chamber (56 X 44 X 49 cm) was divided into a main chamber (34 X 44 X 49 cm) and an escape chamber (22 X 44 X 49 cm). During these tests, the females could retreat to the escape chamber through one of four holes in the Plexiglas divider that separated the main and escape chambers, and through which the larger male was unable to pass. The stimulus male was introduced into the main chamber 5 min before the experimental female was placed in the escape chamber. The test ended after the female received an ejaculation and returned to the main chamber after her postejaculatory refractory period. However, if the female did not leave the main chamber within 60 s following ejaculation, the test was stopped. Alternatively, if the female did not receive an intromission within 15 min or an ejaculation within 20 min after the start of the test, the test was terminated.

During this test, the following behavioral measures were recorded: approach latency (AL, the amount of time it takes the female to cross into the main chamber after being introduced in the escape chamber), mount latency (ML, the time between the female's entry into the main chamber until the first mount), and intromission latency (IL, the time

between the female's entry into the main chamber and the first intromission). Mount return latency (MRL), intromission return latency (IRL), and post-ejaculatory refractory period (PER) refer to the length of time between the female leaving the main chamber following a mount, intromission, and ejaculation, and returning. All latency measures are expressed in seconds. The percentage of times that the female escaped from the male following a mount or intromission was calculated by dividing the number of exits by the number of each copulatory event. The percentage of time the female spent with the male was also calculated by dividing the amount of time the female spent in the main chamber by the total test time. In addition, the sexual receptivity of experimental females was analyzed using the lordosis quotient (LQ). When the female is mounted, she shows a reflexive posture that is characterized by a pronounced arching of the back called lordosis. LQ is calculated by the following formula: (number of lordosis responses during the first 10 mounts/10) X 100, or (number of lordosis responses/number of mounts) X 100 when the number of mounts was smaller than 10 during the pacing test.

General Analyses

Preliminary analyses revealed that the sexual behavior and partner preference exhibited by male and female offspring were not different between those offspring reared by their own mother or by a foster mother (*t*-tests, *p*s = 0.35 – 0.89 for all behaviors examined). For this reason, data from the group of PCB mothers rearing their own PCB pups (uncrossed) were combined with those from the group of PCB dams rearing a litter of

PCB pups that were not her own (crossed). The same was done for the crossed and uncrossed oil groups.

Partner Preference Analyses

2 X 2 ANOVAs (treatment X first or second test) with repeated measures on the second factor were used to test the hypothesis that any exposure to PCB affected the offsprings' partner preference. This was evaluated by comparing the offspring of the oil group to a combined PCB group that included offspring of all groups receiving any PCB exposure. In addition, to evaluate the effects of the three types of PCB exposure against the oil group, 4 X 2 ANOVAs (treatment X first or second test) with repeated measures on the second factor were used to analyze the data. Significant interactions and/or main effects and simple effects were followed by Fisher's PLSD. Differences were considered statistically significant if $p \leq 0.05$.

Sexual Behavior Analyses

If a male did not achieve ejaculation his score was left out of the analysis. For each behavioral measure obtained from experimental animals of both sexes, a *t*-test was used to test the hypothesis that any exposure of the litter to PCB affects the pups' sexual behavior. One-way ANOVAs were also used to evaluate the effects of the four different PCB treatments on behavior and significant F ratios were followed by post hoc tests. All differences were considered statistically significant if $p \leq 0.05$.

RESULTS

Male Partner Preference

The 2 x 2 ANOVA comparing all PCB-exposed animals to the oil-only group over the two preference tests did not reveal significant effects of treatment or significant interactions for the percent of time spent in each chamber (data not shown). However, a significant effect of test was found for percent of time spent in the female chamber [F (1, 33) = 5.33, p = 0.027] and percent of time spent in the male chamber [F (1, 33) = 11.36, p = 0.002], with males spending more time with the female and less time with the male during the second test than during the first test (data not shown).

The ANOVA of the four treatment groups detected a significant interaction between treatment and test for time spent in the stimulus female chamber [F (3, 31) = 3.14, p = 0.039]. For the groups that received both pre- and postnatal exposure to PCB, there was a simple main effect of test (t = -2.41, df = 20, p = 0.026); these animals spent more time with the female during the second partner preference test than the first test (Figure 17). There was no significant simple main effect of treatment on percent of time spent with the female during the first test (Figure 17). However, there was a simple main effect of treatment during the second partner preference test [F (3, 38) = 3.58, p = 0.023]; none of the PCB-treated groups were different from the oil-only group on this second test, but

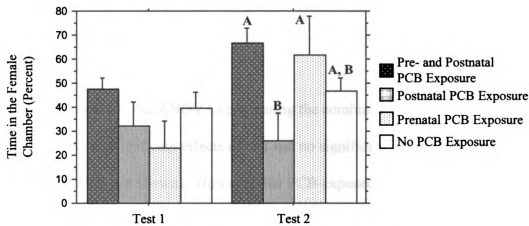


Figure 17. A graph illustrating the differences found with the post hoc analysis that followed the significant interaction between treatment and week for percent of time males spent in the female chamber. There were no significant differences during the first partner preference test. During the second test, there was a significant effect of treatment; the groups sharing the same letter are not significantly different from each other.

males from both groups exposed to PCB prenatally spent more time in the female chamber than those males who received only PCB exposure postnatally (Figure 17).

Female Partner Preference

For all dependent variables, the ANOVAs comparing the combined PCB groups to the oil-only group found no significant effects of test and no significant interactions between treatments and test (data not shown). However, the PCB-exposed group spent significantly more time with the stimulus female [$F(1, 33) = 18.51, p < 0.001$] and significantly less time with the stimulus male [$F(1, 33) = 8.76, p = 0.006$]. Those main effects of PCB exposure are shown in Figures 18 and 19, respectively. There was no effect of PCB exposure on percent of time spent in the middle chamber ($p = 0.90$; data not shown).

The ANOVAs comparing the four treatment groups across the two tests found no significant effects of test and no significant interactions. But there was a significant main effect of treatment on percent of time spent with the stimulus female [$F(3, 31) = 7.60, p < 0.001$]. Post hoc analysis revealed that females receiving postnatal PCB exposure only and those receiving both pre- and postnatal PCB exposure spent more time with the stimulus female than the oil group (Figure 20). For percent of time spent with the stimulus male, the ANOVA detected a significant main effect of treatment [$F(3, 31) = 6.22, p = 0.002$] and post hoc tests showed that females that received both pre- and post-

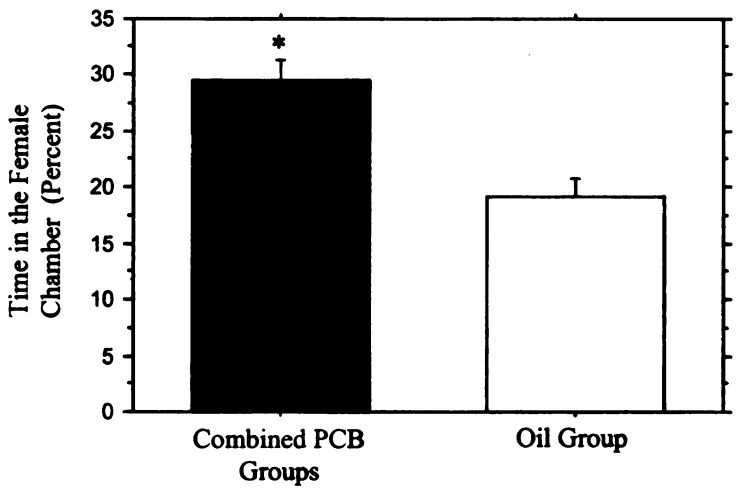


Figure 18. A comparison of the combined PCB groups to the oil only group found a significant difference (*) in the amount of time females spent with the stimulus female, with the PCB- exposed animals spending more time in the female chamber than the oil animals.

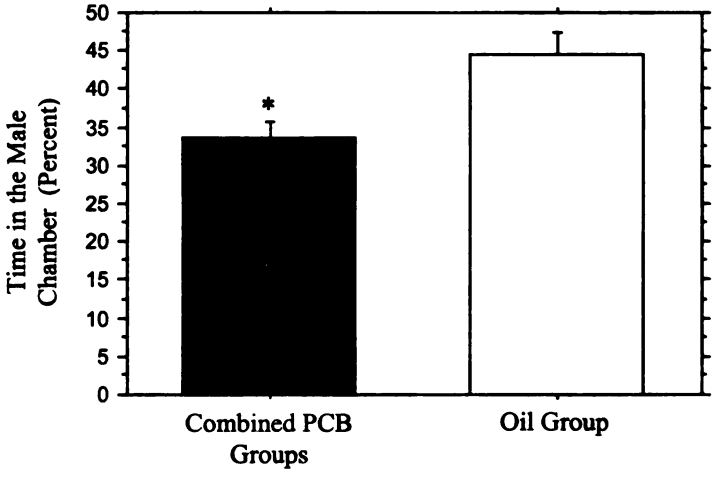


Figure 19. A comparison of the combined PCB groups to the oil only group found a significant difference (*) in the amount of time females spent with the stimulus male, with the PCB- exposed animals spending less time in the male chamber than the oil animals.

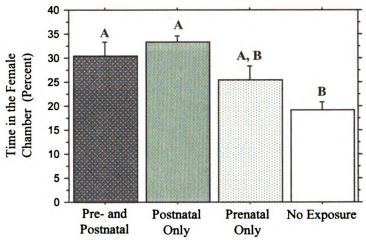


Figure 20. The ANOVA of the data examining the four treatment groups found a significant main effect of treatment on percent of time females spent with the stimulus female; the groups sharing the same letter are not significantly different from each other.

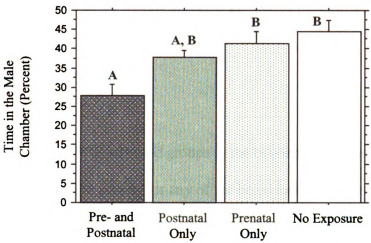


Figure 21. The ANOVA of the data examining the four treatment groups found a significant main effect of treatment on percent of time females spent with the stimulus male; the groups sharing the same letter are not significantly different from each other.

natal PCB exposure spent less time with the male than those females that received either only prenatal or no PCB exposure (Figure 21). There was no effect of treatment on percent of time spent in the middle chamber ($p = 0.16$; data not shown).

Male-Paced Sexual Behavior

A comparison of all PCB-exposed groups to the oil-only group revealed no significant differences between the groups for any of the behavioral measures that were analyzed. One-way ANOVAs of the four treatment groups also failed to reach significance for all behaviors examined (Table 2).

Female-Paced Sexual Behavior

A comparison of all PCB-exposed groups to the oil-only group revealed no significant differences between the groups for any of the sexual behavior measures that were investigated. One-way ANOVAs of the four treatment groups also failed to reach significance (Table 3).

Table 2
Exposure to PCBs and male sexual behavior

	PCB/PCB (n = 8)	PCB/oil (n = 8)	oil/PCB (n = 8)	oil only (n = 8)
# M	13.1 ± 1.2	12.0 ± 2.4	15.3 ± 4.1	11.1 ± 1.7
# I	14.4 ± 1.5	10.4 ± 1.1	11.5 ± 3.5	11.5 ± 3.9
ML (s)	40.6 ± 14.3	106.6 ± 50.8	64.1 ± 19.8	140.6 ± 53.9
IL (s)	49.5 ± 10.9	187.1 ± 75.4	74.6 ± 32.9	219.4 ± 80.5
EL (s)	483.2 ± 50.1	564.6 ± 59.2	535.1 ± 76.5	376.5 ± 83.7
III (s)	41.3 ± 4.0	43.4 ± 2.3	44.2 ± 5.3	45.0 ± 8.4
PEI (s)	301.1 ± 33.7	260.8 ± 63.1	177.3 ± 27.5	220.7 ± 30.1
CE (%)	52.0	58.4	52.5	59.3

The data for the first seven measures are presented as mean ± S.E.M. No significant differences between groups were detected with p values ranging from 0.32 – 0.88. See text for statistical tests. PCB/PCB = prenatally PCB-exposed pups reared by PCB-treated dams; PCB/oil = prenatally oil-exposed pups reared by PCB-treated dams; oil/PCB = prenatally PCB-exposed pups reared by oil-treated dams; pups only exposed to oil; # M = number of mounts; # I = number of intromissions; ML = mount latency; IL = intromission latency; EL = ejaculation latency; III = inter-intromission interval; PEI = postejaculatory interval; CE = copulatory efficiency; n = number of litters.

Table 3
Exposure to PCBs and female sexual behavior

	PCB/PCB (n = 8)	PCB/oil (n = 8)	oil/PCB (n = 8)	oil only (n = 8)
AL (s)	4.6 ± 1.0	10.5 ± 4.3	6.1 ± 0.3	8.1 ± 1.1
ML (s)	65.4 ± 28.6	128.5 ± 112.7	59.6 ± 20.9	86.7 ± 33.0
IL (s)	37.8 ± 27.0	140.0 ± 111.2	120.7 ± 65.2	89.8 ± 38.9
MRL (s)	8.5 ± 2.0	27.0 ± 18.1	5.9 ± 2.3	15.8 ± 7.5
IRL (s)	30.9 ± 3.5	35.6 ± 2.9	14.6 ± 5.7	39.0 ± 13.7
PER (s)	67.7 ± 11.8	107.6 ± 26.7	65.1 ± 12.8	114.9 ± 25.7
LQ (%)	77.9 ± 4.8	78.3 ± 4.9	75.0 ± 7.2	71.9 ± 7.4

The data for the measures are presented as mean ± S.E.M. No significant differences between groups were detected with p values ranging from 0.22 – 0.74. See text for statistical tests. PCB/PCB = prenatally PCB-exposed pups reared by PCB-treated dams; PCB/oil = prenatally oil-exposed pups reared by PCB-treated dams; oil/PCB = prenatally PCB-exposed pups reared by oil-treated dams; pups only exposed to oil; AL = approach latency; ML = mount latency; IL = intromission latency; MRL = mount return latency; IRL = intromission return latency; PER = postejaculatory refractory period; LQ = lordosis quotient; n = number of litters.

DISCUSSION

It is important to note that although we refer to our testing paradigm as a "partner preference" situation, and even though sexual interaction was allowed between the experimental and stimulus animals, we do not imply that we tested the animals for their "mate choice." We determined with whom the experimental animal would rather spend time when given the choice between a sexually active male or a hormone-primed, sexually receptive female when sexual interaction was allowed to take place. Preferring to spend time with an animal of the same sex to one of the opposite sex does not imply that the experimental subject would prefer to mate with an animal of the same sex. It means that given the choice, and under these conditions, the experimental animal would rather spend time in the vicinity of one stimulus animal over another.

Male Partner Preference

The comparison of the combined PCB groups with the oil group showed that regardless of treatment, males spent more time with the stimulus female after receiving sexual experience. However, analysis of the four separate treatment groups revealed that this overall increase in female preference during the second partner preference test was mainly due to the increase in preference exhibited by males that received double PCB exposure. That is, no other group's preference changed significantly after sexual experience. This enhanced responsiveness to the effects of sexual experience on preference for the female was the only feature that differentiated any PCB-exposed group

from the oil controls. An increase in preference for the female induced by sexual experience has been reported for control male rats (Brand and Slob, 1991; Bakker *et al.*, 1996), but the mechanisms responsible for this effect of experience are not known. Consequently, it is difficult to generate hypotheses to explain why we did not see this increase in the control group or how it may be augmented by perinatal exposure to PCB 77. In the present study, there were no differences across groups on any aspect of male sexual behavior; therefore it is unlikely that differences in quantity or quality of sexual experience were responsible for the group differences.

Female Partner Preference

Females exposed to PCB perinatally spent more time with the stimulus female and less time with the stimulus male than females that were not exposed to PCB. Comparison of the combined PCB groups to the oil group revealed that any exposure to PCB altered the amount of time females spent with both stimulus animals compared to the control females. With respect to time spent with the female stimulus, the more detailed analysis revealed that females reared by dams that had been treated with PCB while pregnant spent more time in the vicinity of a sexually receptive female than controls. In addition, those females that received double exposure during development also showed a significant reduction in the time spent with the male when compared to controls.

The altered partner preference displayed by females who were reared by PCB-exposed dams may be partially attributed to the changes in the care provided by their mothers.

Analysis of some aspects of the maternal care received by these animals (see Chapter 3) revealed a pattern of licking and grooming behavior in the dams that was very similar to the females' partner preference profile. PCB-exposed dams displayed an increased amount of pup-directed LG, and the females from those dams exhibited an increased preference for the stimulus female. In addition, while the differences in pup-directed LG between the four treatment groups was not statistically significant (Chapter 3), the three PCB-exposed groups appeared to spend more time engaged in LG than the oil-only dams.

A correlation was run to test the hypothesis that the amount of LG that the PCB-exposed females received was correlated to the increased percent of time they spent with the stimulus female and decreased amount of time spent with the stimulus male. The percent of time that each PCB-exposed female spent with the stimulus male and stimulus female was compared to the average amount of LG that her litter received over the four postnatal days. Interestingly, the correlation did not reveal any significant effects (rs = -0.01 – 0.29). It is possible that a correlation does exist but is obscured by other factors. For example, the correlational analysis used the average amount of LG that the entire litter received over the four postnatal days that were examined. Our procedural methods do not allow us to examine the specific amount of LG that each female received on each postnatal day. Thus, although the mean amount of LG that the litter received over all four postnatal days does not correlate with the percent of time PCB-exposed females spent with the stimulus animals, the amount of LG each individual female received may correlate to the percent of time spent with the stimulus animals.

PCBs and other environmental contaminants have been detected in the milk of rats (Crofton *et al.*, 2000; You *et al.*, 1999) and humans (Dewailly *et al.*, 1991; Schulte and Malisch, 1984; Jensen, 1987; Noren and Lunden, 1990) after exposure to the toxin, indicating the possibility of lactational transfer to the offspring. Thus, in addition to possible maternal influences, the offsprings' change in partner preference may be due to direct effects of the PCBs that they received from the dam's milk. PCB exposure has also been found to have greater developmental effects when the animal is exposed postnatally rather than prenatally. For example, exposure to Aroclor 1254 caused permanent low-frequency hearing deficits and hypothyroxinemia in rats who were exposed postnatally, whereas offspring who received only prenatal exposure were not significantly affected (Crofton *et al.*, 2000). Thus, it is not surprising that the females who are reared by PCB-treated dams are different from females reared by oil-treated dams.

It is possible that the preference PCB-exposed females exhibit for the stimulus females is not be the result of increased sexual motivation but increased fearfulness associated with the male. Conceivably, PCB-exposed females may be interpreting novel situations as fearful, particularly those in which a sexually active male is tethered in one chamber and where it is possible to escape to a compartment that contains a more familiar, less novel animal (i.e. another female). The PCB-treated females may not be attracted to the stimulus female as a result of sexual motivation, but because another female is a more familiar or less novel stimulus. However, our findings that PCB-exposed offspring received an increased amount of LG, and the work conducted by Meaney and colleagues

(Liu *et al.*, 1997; Caldji *et al.*, 1998), would indicate that this is not likely to be the case. Females that receive an increased amount of LG from their mothers tend to be less fearful and less reactive to novel situations. On the other hand, the increased amount of LG demonstrated by PCB-treated dams did not reach the level of LG exhibited by the dams in Meaney's studies. Therefore, the offspring reared by PCB-exposed dams may not have received the amount of LG that is required in order to decrease the females' level of fear. To test this hypothesis, one would need only to measure the stress response of these females when they are placed in the vicinity of a stimulus male. If the stress response is within the normal range, or similar to that of control females, then the PCB-exposed females are not spending more time in the stimulus females' chamber because of an increased fear of the male.

It is also possible that the reason PCB-exposed females spent more time in the stimulus females' chamber is that the PCB-exposed females are more sexually attracted to the stimulus females. One way to determine if PCB exposure is increasing the females' sexual preference for other females is to measure the experimental animals' proceptivity level in either a partner preference or female-female pacing situation. If PCB exposure increases the females' sexual preference for females, then we would expect the affected individuals to show an increase in proceptive behaviors such as ear wiggling, hopping and darting. An increase in these behaviors would lend evidence to the possibility that PCB treatment has altered the females' choice for a sexual partner. This hypothesis is currently being tested in our laboratory by further analysis of the video recordings of the partner preference tests. The possibility that PCB-exposed females are spending more

time with the stimulus females and less time with the males because of a decrease in receptivity can be ruled out, since the lordosis quotients of the PCB- and oil-exposed groups are not different.

While PCB affected the stimulus preference of both sexes, the effects were more salient for the females. This difference may be due in part to the different procedures employed for the two sexes. Experimental males remained intact for the tests, while experimental females were gonadectomized and brought into receptivity using exogenous estrogen and progesterone. It is possible that the PCBs are affecting the males and females similarly, but the males are able to compensate for any changes caused by the PCBs because of their functioning testes.

Male-Paced Sexual Behavior

In contrast to the changes seen in male partner preference, gestational and/or lactational exposure to PCB 77 did not alter male sexual behavior in the pacing paradigm. Comparison of the combined PCB-exposed groups to the oil-only group as well as comparison of the four individual treatment groups did not reveal any significant differences. These findings parallel those of previous experiments in which a single oral dose of PCB 77 on gestation day 15 (Faqi *et al.*, 1998) and multiple intraperitoneal doses on gestation days 7 – 18 (Wang *et al.*, 2002) failed to alter male sexual behavior.

It is also important to note that the increased amount of pup-directed LG provided by PCB-treated dams may serve to buffer or compensate for some of the toxic effects that the offspring may otherwise incur as a result of exposure to the contaminant. As mentioned in the Discussion section of Chapter Three, manipulations that increase licking and grooming (e.g. neonatal handling) have been shown to protect the offspring from the effects of early-life insults including fetal ethanol exposure (Lee and Rabe, 1999) and cerebral hypoxia (Chou *et al.*, 2001). Previous research has documented the importance of maternal behavior, in particular licking and grooming, in the development of male sexual behavior (Moore, 1984; Moore, 1995). Since PCB-exposed dams exhibited an increased amount of pup-directed LG, it is possible that some developmental effects that would otherwise be seen in the pups as a result of PCB exposure may be lessened because of the increase in licking and grooming that is exhibited by the PCB-exposed groups.

There are a few experiments that could be conducted in order to test whether the increased LG male pups receive from PCB-exposed mothers reduces some of the effects that PCBs might otherwise have of male sexual behavior. First, PCB-exposed male offspring could be fostered to mothers that show either significantly increased or significantly decreased LG. If males reared by a low LG dam demonstrate altered sexual behavior compared to those males reared by a high LG dam, then it is likely that we did not see effects of PCB on male sexual behavior in our study because the increased LG exhibited by the dams attenuated the effects of the toxin on the offspring.

Another possible experiment to determine the role of LG in PCB-affected male sexual behavior would be to administer zinc sulfate to PCB-treated dams in order to render them anosmic. This procedure has been shown to significantly decrease the amount of licking that the pups receive (Moore and Power, 1992; Moore *et al.*, 1992). However, there are additional considerations that need to be taken into account if this method is employed. For example, nearly eliminating the amount of stimulation provided by the dam as a result of her being anosmic has been shown to affect the display of male sexual behavior (Moore, 1992). Therefore, any changes seen in male sexual behavior exhibited by PCB-exposed offspring reared by anosmic dams cannot be conclusively attributed to the PCB.

Finally, an experiment that identifies the exact amount of LG that each PCB-exposed pup receives would help to elucidate the possible role of LG in attenuating the effects of PCB on male sexual behavior. Our design did not allow for individual examination of the amount of maternal behavior each offspring received. While we can identify if a dam demonstrated a high or low amount of LG, the amount of individual stimulation each pup receives often varies within the litter. If we could correlate the amount of LG that each pup receives from PCB-exposed dams to his sexual behavior performance, we would be able to identify if increased LG affected this behavior.

Female-Paced Sexual Behavior

While PCB 77 affected female partner preference behavior, we did not see any significant effects of the toxin on the females' copulatory behavior. No significant differences were

found in any behavioral measures when comparing the combined PCB-exposed groups to the oil-only group, nor when comparing all four treatment groups to one another. Previous research from our laboratory using commercial PCB mixtures demonstrated significant changes in female receptivity and sexual behavior after the offspring had been exposed to the mixture in the early prenatal (Chung *et al.*, 2001) and the perinatal period (Chung and Clemens, 1999). However, it is difficult to compare our current findings to those of earlier experiments because they used mixtures of PCBs that were comprised of both coplanar (the class of PCBs of which PCB 77 is a part) and ortho-substituted PCBs, whereas the current study employed a single PCB congener.

In a study in which PCB 77 was delivered during the same gestation days as used in the current experiment but utilizing a different route of administration (IP), Wang *et al.* (2002) describe reduced female sexual behavior and receptivity. The discrepancies between their results and those reported here could be due to the differential metabolism of the PCB as a result of different routes of administration. Hany *et al.* (1999a) examined the relative amounts of PCB congeners that remained in various tissue types after subcutaneous treatment of the dam on GD 7 – 18. The concentration of PCB 77 that remained in tissue was extremely small, indicating that PCB 77 is metabolized quickly when the toxin is administered subcutaneously. Although there are no data demonstrating the how quickly the PCB would be metabolized after IP administration, there is the possibility that the toxin would be degraded more slowly. Thus, the differences between the female sexual data presented here and those from Wang *et al.* may be explained by the possibility that PCB 77 has a greater effect on female sexual

behavior when it is delivered IP because it is not degraded as quickly as when the contaminant is administered subcutaneously.

Summary

While perinatal PCB exposure did not affect sexual behavior, it did alter female and male partner preference. There was an interaction between treatment and test for PCB-exposed males with regards to their stimulus animal preference. Treatment with PCB prenatally increased the effects of sexual experience on the males' preference for a stimulus female compared to postnatal PCB treatment. Any exposure to PCB altered the females' preference; pre- and/or postnatal exposure increased the amount of time females spent with the stimulus female and decreased the amount of time spent with the stimulus male. The changes seen in the females may be due in part to altered maternal care provided to the offspring as a result of the PCB exposure their mothers received while pregnant. Specifically, the pattern of licking and grooming exhibited by PCB-exposed dams is similar to the preferences exhibited by the female offspring. While this does not explain the pattern of behavior for the males, it is interesting that by increasing the amount of licking and grooming the females receive (i.e., masculinizing the amount of stimulation) the females' preference for both stimulus males and females is masculinized.

Chapter 6. General Discussion

For decades the traditional toxicological paradigm used to evaluate the effects of a toxin on a developing organism involved treating pregnant rats with a specific contaminant and examining the offspring. In light of the work presented in this dissertation as well as that conducted by Simmons *et al.*, it is clear that this traditional paradigm needs to be revised to include an analysis of maternal behavior. Taking into consideration the accumulating evidence in recent years, it is essential that we control for the effects on the mother when evaluating whether treatment effects on the offspring are a direct result of the treatment on the developing organism or a reflection of alterations in maternal care, or some combination thereof.

In the traditional paradigm, treatment of the dam is simply considered an avenue through which toxins can be delivered to the developing embryo (for example, see Loeffler and Peterson, 1999; Simanainen *et al.*, 2004). Clearly, such exposure is not specific to the pups and effects of the contaminant on the dam must be factored into the eventual outcome. Changes in maternal care are often accompanied by changes in the offsprings' physiology and/or behavior. Without taking into account possible changes in the behavior of the dam, we cannot state with certainty that the effects seen in the offspring are due to the direct effect of the toxin on the pups as is frequently reported (e.g. Loeffler and Peterson, 1999).

As early as the 1960s the impact of neonatal stimulation on the development of the litter was clearly established. At that time, the authors noted that their daily handling of the litter changed various aspects of the pups' behavior when they were tested as adults (Levine and Thoman, 1969; Thoman and Levine, 1969). The authors also identified changes in the dam that were due to handling of the pups (Levine and Thoman, 1969). At that time, however, the mechanisms responsible for these changes were not known. It was not until 30 years later that scientists linked handling of the pups to specific changes in maternal care. Meaney and colleagues (Liu *et al.*, 1997) identified an increase in the amount of pup-directed licking and grooming provided by the dams of handled offspring, and linked these changes in maternal care to a variety of physiological and behavioral alterations seen in the offspring.

In this thesis, we have demonstrated not only that PCB treatment to rats during gestation alters the care provided by the exposed dams, but that the behavior of the dams can also be altered in response to rearing prenatally PCB-exposed offspring (Chapters 3 and 4). Although the dynamic interactions that take place between a mother and her pups are not new, this is an area which has been virtually ignored in the field of toxicology. Additional analyses must be conducted to further address how early treatments can modify adult behavior via direct or indirect action of the treatment on both the offspring and their mother.

We have also demonstrated effects of PCB exposure on partner preference behavior in female rats (Chapter 5). New hypotheses arose out of our analysis of offspring partner

preference that warrant further consideration. For example, the sexual motivation of the PCB-exposed females may have been altered in the direction of preferring stimulus females rather than males. The additional analyses will include correlating offspring behavior to the amount of pup-directed licking and grooming that the offspring received as neonates to evaluate potentially confounding effect of PCB-induced alterations in maternal care on the offspring.

Environmental contamination with man-made chemicals is unfortunately a fact of modern life and is a global problem. Even though PCBs are no longer produced they continue to enter and exist in the ecosystem and move throughout the world, threatening the existence of both human and non-human populations. Saving these populations from extinction and protecting ourselves from the effects of exposure requires an understanding both of the global dynamics of PCBs as well as how these contaminants affect the organisms with which they come in contact. Since it is clear that we can not fully protect ourselves against PCB exposure, we must find ways to attenuate the effects. While there is no miracle "cure," there are some measures that may be taken in order to help ameliorate the effects of PCB exposure. For instance, environmental enrichment, defined as "a combination of complex inanimate objects and social stimulation" (Guilarte *et al.*, 2003), has been shown to attenuate deficits produced by developmental lead exposure (Guilarte *et al.*, 2003). There also have been several reports demonstrating the efficacy of early intensive behavioral therapy, sensory stimulation and environmental enrichment in reversing (or overcoming) some of the core autistic symptoms (Schneider *et al.*, 2006; Harris *et al.*, 1991; Scheinkopf and Siegel, 1998; Ozonoff and Cathcart,

1998), that surprisingly seem also to be triggered by early PCB exposure. Thus, it is possible that employing similar techniques to children exposed to PCBs developmentally may help to reduce the observed deficits. Importantly, these interventions have the greatest effect when the disorders are detected early (Filipek *et al.*, 2000; Sandler *et al.*, 2001), stressing the importance of identifying possible effects and symptoms of PCB exposure in order to better diagnose and treat the deficits early on.

This thesis is a small step when considering the enormity of the problem. However, it does establish that the earlier paradigms used to analyze the effects of toxins on developing organisms needs to be expanded to include consideration of the complex interaction between the contaminant and the dynamic mother-infant relationship.

APPENDICES

Appendix A: The effect of developmental PCB exposure on open field behavior.

This experiment was conducted in order to test the hypothesis that developmental PCB exposure affected the animals' display of fear in an open, well-lit arena. The animals that were tested in this study were born to the dams from Chapter Two, Experiment Two in which the pregnant rats were treated with a subcutaneous dose of PCB 77 during gestation and given an untreated cookie immediately after the injection. The offspring were weaned from the dam on postnatal day (PND) 23 and housed in same-sex pairs.

Testing began at 60 and 90 days of age for females and males, respectively. All animals were left intact. The open field apparatus was made of opaque Perspex and was 120 X 120 cm. Lines were drawn on the floor of the apparatus using a large permanent marker in order to determine the mobility of the animal. The experimental animal was placed in a small, black, Perspex box and put in the corner of the apparatus before the test began. The box was completely enclosed save for one of the walls that the experimenter could remove at the start of the test to let the animal out. Tests lasted 10 mins from the time at which the wall of the black box was removed. The following parameters were recorded: latency to emerge from the black box, amount of time spent in the field, percent of time spent in the perimeter compared to the center of the field, number of lines crossed, number of boluses in the black box, and number of boluses in the field.

None of the males exposed to 4 mg/kg PCB were still alive when testing began on PND 90. Thus our analysis included females from the 2 and 4 mg/kg groups and males from

the 2 mg/kg group. Our analysis did not reveal any significant main effects of treatment on any of the parameters measured. Thus, it appears that developmental exposure to 2 or 4 mg/kg does not affect the animals' performance in the open field. However, this does not mean that the animals' stress or fear responses are not altered by exposure to PCB, but that the behaviors measured with our experiment were not affected. Examining the animals' responses in a different testing situation designed to analyze different behaviors would help to address this question of whether or not the animals' fear responses are affected by PCB exposure (e.g., measuring the animal's stress response in a restraint situation).

Appendix B: The effect of developmental estrogen (E) on maternal behavior.

Since PCB has been shown to act as an estrogen, we designed an experiment to see if PCB 77 was altering maternal behavior by acting estrogenically. Pregnant dams were to be treated with varying amounts of E during gestation, after which their maternal behavior would be recorded on the same PNDs as those examined in the PCB studies (PND 1, 2, 4 and 6) and compared to the maternal behavior exhibited by dams exposed to PCB 77.

We used a total of six different doses over the duration of our experiment, yielding an increase in serum levels of E to approximately 110 pg/ml, 55 pg/ml, 35 pg/ml, 25 pg/ml, 20 pg/ml and 10 pg/ml (exposure via silastic implants, approximations from Bridges, 1984). However, no dams carried their fetuses to full term; the pregnancies of all dams were aborted or the embryos resorbed. It is likely that our doses of E were too high for the dam to maintain the pregnancy. Throughout the pregnancy, E remains low and rises only at the end before parturition (for example see Siegel and Rosenblatt, 1975). The increased level of E produced by our implants may have signaled to the dam's body that her current hormonal conditions were not optimal and aborted the pregnancy.

Appendix C: The effect of PCB-exposed dams on offspring nipple attachment.

In the experiment examining the effects of 2 mg/kg PCB on maternal care, we found a decrease in the amount of time the dams spent in the high crouch nursing posture after PCB exposure. Since the dam cannot engage in high crouch nursing unless she receives the appropriate amount of ventral stimulation (including rooting around for and attaching to a nipple), we wondered if the offspring being reared by PCB-exposed dams found them aversive and were not motivated to attach to their nipples.

In order to test this, we treated dams with 3 mg/kg PCB 77 during gestation. On the day of birth, dams and litters were cross-fostered to make the following four groups: PCB-treated dams rearing PCB-exposed offspring, PCB-treated dams rearing oil-exposed offspring, oil-treated dams rearing PCB-exposed offspring, and oil-treated dams rearing oil-exposed offspring. On PND 7, litters were removed and placed in an incubator for 2 hours to induce hunger in the pups. At the end of the 2 hr period, dams were anesthetized and placed on her side. Pups were placed 2 cm from the dam, one pup close to the anterior and one close to the posterior ends of her ventrum. Tests were stopped either when both pups attached to the nipples or after 3 min had elapsed. Three trials were run per dam, one trial with each group of pups. Dams were not tested with the litters they reared, and the order of presentation of pups was randomly varied from dam to dam.

Very few pups attached to the dam within the time limits of the test, so we were not able to run any analyses to test the hypothesis that PCB-exposed dams spent less time engaged

in high crouch nursing because the offspring found the dams to be less attractive, or aversive, and spent less time suckling.

Appendix D: The effect of PCB-treatment on expression of *c-fos* in maternal brain areas.

In order to see if PCB-treatment to the dams or the pups altered the expression of *c-fos* in maternal brain areas, dams were treated with 3 mg/kg PCB 77 during gestation. On the day of birth, dams and litters were cross-fostered to make the four exposure groups. On PND 4, all pups were removed from the dams and given back during the light cycle 48 hr later. This was done in order to induce the expression of maternal behavior in the hopes of also inducing expression of *c-fos*. The behavior of the dams was recorded for 2 hr after the pups were replaced for confirmation of behavioral expression and to perhaps compare the amount of behavior displayed with the expression of *c-fos*.

A total of 5 of the 14 dams tested responded maternally after replacement of the pups (Table 4). The reasons for this remain unclear; the paradigm we followed has often been used to successfully induce maternal behavior. However, since we were not able to induce maternal behavior, we did not examine the brains for PCB-induced alterations in *fos* expression.

Table 4.
Number of Dams Responding Maternally.

Treatment of Dam	Treatment of Pups	Number of Dams Responding Maternally
PCB	PCB	2 out of 3
PCB	oil	1 out of 3
oil	PCB	1 out of 4
oil	oil	1 out of 4

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