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INDUCTION OF HEPATIC CYTOCHROME P450 AND EXAMINATION OF OVARIAN STEROID RECEPTOR HOMEOSTASIS IN THREE GENERATIONS OF MINK CONSUMING PCB-CONTAMINATED CARP FROM SAGINAW BAY, LAKE HURON

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By

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ABSTRACT

INDUCTION OF HEPATIC CYTOCHROME P450 AND EXAMINATION OF OVARIAN STEROID RECEPTOR HOMEOSTASIS IN THREE GENERATIONS OF MINK CONSUMING PCB-CONTAMINATED CARP FROM SAGINAW BAY, LAKE HURON

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The mink population of the Great Lakes basin has been declining over the last six decades. Although part of this decline may be due to increasing use of land previously part of the mink habitat, a larger cause is the contamination of the Great Lakes environment with halogenated aromatic hydrocarbons such as polychlorinated biphenyls, polybrominated biphenyls, polychlorinated dibenzodioxins, and polychlorinated dibenzofurans. Mink were discovered to be extremely sensitive to these contaminants, particularly in terms of their reproductive toxicity. A feeding study was conducted in which adult mink were fed one of three diets containing Saginaw Bay carp to provide 0.25, 0.5, or 1.0 ppm polychlorinated biphenyls. A fourth group of mink was fed a control diet containing ocean fish, which provided 0.0 ppm polychlorinated biphenyls. Mink were bred after 3 months consumption of the diet, and half of the adults and kits were transferred to the control diet at weaning of the kits. The mink were bred again during the following season, after 10 or 13 months on the diets. The animals were sacrificed at weaning of the second generation of kits. In order to determine the potential for a biomarker of halogenated aromatic hydrocarbon consumption in mink, cytochrome P450 enzyme activity was measured in livers of 217 mink which had consumed diets containing Great Lakes fish for up to eighteen months. The activity of cytochrome P450IA1 had a greater dose-response relationship to PCB consumption than did the other cytochrome P450 enzyme activities measured. Hepatic and uterine estrogen receptor

concentrations were measured in tissues from the 143 female mink to determine if the reproductive toxicity observed in mink may be due to decreased estrogen receptor concentrations, and thus an inability to detect varying concentrations of estrogen in the blood. In general, the hepatic estrogen receptor concentration decreased in a dose-dependent manner with increasing PCB concentration in the diet. Uterine estrogen receptor concentrations did not change with increasing dietary PCB concentrations. Uterine progesterone receptor concentration was also unaffected.

This is dedicated with love and thanks to my parents.

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INTRODUCTION

Quite often, the focus of a toxicological investigation of a chemical in industry or in the environment is related to the lethal or carcinogenic dose, and the latency period before the appearance of either of these responses. However, chemicals often have other toxicological endpoints that are less investigated. One such "neglected" endpoint is reproductive decrease or failure. However, the ability of industrial chemicals and environmental contaminants to disrupt reproduction and other aspects of the endocrine system is receiving more interest (Crisp *et al.*, 1998). As the environment is improved so that exposure to pollutants changes from high dose, acute exposure and acute toxicity to low dose, chronic exposure, the potential for chronic non-lethal toxicity increases.

Among the endocrine disruptors being investigated is the group of halogenated aromatic hydrocarbons. These compounds, which include polychlorinated dibenzodioxins, polychlorinated biphenyls, and polybrominated biphenyls, have been shown to decrease reproductive ability in different animals (Fox, 1991). Although in utero exposure to halogenated aromatic hydrocarbons may alter reproductive behavior in males in some species, the main impact on reproductive ability appears to occur in the female. This is seen as increased fetal resorption during gestation, decreased numbers of young per litter, and increased post-natal death of the young (Aulerich *et al.*, 1973; Brezner *et al.*, 1984; Wren, 1987b).

The mechanism by which halogenated aromatic hydrocarbons cause reproductive toxicity appears to be mediated through interference with the ability of estrogen to regulate the reproductive process. In cultured cells, decreases in both estrogen concentration and estrogen receptor concentrations have been observed (Spink *et al.*, 1990, 1991; Harris *et al.*, 1990). In the whole animal, the effects produced include decreased estrogen receptor concentrations in liver and uterus, decreased uterine wet weight, and decreased expression of estrogen-stimulated proteins such as epidermal growth factor, insulin-like growth factor, and *c-fos* (Astroff *et al.*, 1990a, 1991).

It has been hypothesized that halogenated aromatic hydrocarbons decrease the concentration of estrogen receptors in the uterus, and possibly in other estrogen-responsive tissues as well. By this mechanism, halogenated aromatic hydrocarbons could decrease the ability of those tissues to respond to increasing amounts of estrogen such as those seen prior to ovulation. Decreased responsiveness of the tissues to respond to estrogen fluctuations would account for the decreased ovulation, decreased retention of fetuses, and decreased uterine wet weight that are observed in animals treated with halogenated aromatic hydrocarbons.

Mink have been shown to be extremely sensitive to the reproductive toxicity of halogenated aromatic hydrocarbons, and in fact show decreased reproduction when consuming polychlorinated biphenyls at concentrations below those currently found in Great Lakes fish (Wren *et al.*, 1987a, 1987b; Bleavins *et al.*, 1980). As mink live in the Great Lakes basin and scavenge dead fish from shorelines as well as catching fish from

streams, rivers, and lakes, their exposure to polychlorinated biphenyls through fish consumption may account for the decreasing numbers of mink observed in contaminated areas. This decrease was by as much as 50% in some areas (Wren, 1991).

To examine the potential mechanism by which mink reproduction is affected by polychlorinated biphenyls, adult mink were fed diets containing Saginaw Bay carp which provided up to 1.0 ppm polychlorinated biphenyls. This dietary PCB concentration was chosen because 1) it is below the PCB concentration that has been previously shown to be lethal to adult mink; and 2) it approximates the amount of PCBs that mink would consume in the wild if 30% of their diet came from Saginaw Bay carp. Mink were bred after four months Saginaw Bay carp consumption, and half of the adults and kits were transferred to the control diet at weaning of the kits. The mink were bred again during the following reproductive season, resulting in three generations of mink (P_1, F_1, and F_2) being exposed to PCBs via gestational, lactational, and/or dietary exposure for up to 16 months. If it is assumed that feed consumption by an adult mink is 100g per day, that adult male mink were fed PCB-containing diets for 475 days, and that adult female mink were fed PCB-containing diets for 555 days, then adult males in the 1.0 ppm dietary group consumed approximately 47.5 mg PCBs while adult females consumed 55.5 mg PCBs over the duration of the experiment.

The objectives of the present research were to:

1) determine whether consumption of polychlorinated biphenyls in Saginaw Bay carp would cause some biochemical change, such as enzyme induction, that could be used in

wild-trapped mink to indicate the extent of environmental contamination with polychlorinated biphenyls. It was hypothesized that one or more hepatic cytochrome P450 enzymes would be induced in a dose-dependent manner with increasing consumption of Saginaw Bay carp and polychlorinated biphenyls. This cytochrome P450 enzyme could then be further investigated in other studies to determine its applicability to exposure determination in wild-trapped mink.

2) examine the effect of Saginaw Bay carp consumption by female mink on hepatic and uterine estrogen receptor concentrations. Hepatic tissue was used for estrogen receptor concentration measurement as hepatic tissue is more abundant and thus easier in which to measure biochemical parameters than the much smaller uterus. Additionally, PCBs are stored in hepatic tissue, and thus if PCBs cause a decrease in estrogen receptor concentration in the mink, it is quite likely to create this effect in the liver. The hypothesis to be tested was that hepatic and uterine estrogen receptor concentrations would decrease with increasing polychlorinated biphenyl consumption. Decreasing concentrations of estrogen receptor in normally estrogen-responsive tissues would remove the ability of those tissues to respond to estradiol, perhaps leading to the decreased reproduction seen in polychlorinated biphenyl-exposed animals such as the mink.

3) measure the effect of polychlorinated biphenyl consumption on the concentration of a model estrogen-regulated gene. One such gene encodes the progesterone receptor, the concentration of which is up-regulated by ligand-bound estrogen receptor. If estrogen

receptor concentrations are decreased, it would be expected that the concentration of estrogen-regulated proteins would either remain stable or would decrease. For this objective, the hypothesis was that progesterone receptor concentration would decrease with increasing consumption of Saginw Bay carp by female mink.

These three hypotheses were tested by measuring the activity of three hepatic cytochrome P450 enzymes, and by quantifying hepatic estrogen receptor and uterine estrogen and progesterone receptors in mink fed Saginaw Bay carp.

Chapter 1

LITERATURE REVIEW

Section 1

Overview of Environmental Contamination

During the latter half of the 20th century, man's impact on the environment has become both more serious and better understood. For example, the ability of various accidentally- released and deliberately-used chemicals to affect human and wildlife health and reproduction has generally become understood after releasing those chemicals into the environment. Often this information is the result of observing deleterious effects in exposed populations, then correlating the observations to the offending contaminants. This includes epidemiological studies in humans in areas such as the Great Lakes, as well as both retrospective and prospective studies in wildlife (fish-eating birds, marine mammals such as seals, and terrestrial mammals such as the mink).

The concentration of industry around the Great Lakes, the distribution of wildlife, and the wide use of the Great Lakes area for recreational purposes brings together contaminants, animals, and people in a way that will allow spread of contamination from the environment to primary carnivores in the ecosystem such as mink and man.

Section 2

Halogenated Aromatic Hydrocarbons

One group of compounds to have received increasing attention in the last 30 years is the halogenated aromatic hydrocarbons (HAHs). The toxicity of these compounds was in large part understood only after HAHs had been used and allowed to enter the environment. The HAH group includes polychlorinated biphenyls (PCBs), polybrominated biphenyls (PBBs), polychlorinated dibenzofurans (PCDFs), and polychlorinated dibenzo-p-dioxins (PCDDs), commonly called dioxins. However, as the experiments described in this dissertation discuss Great Lakes contaminants and their effects only in terms of PCBs, the other types of HAHs will not be discussed here. Solutions of PCBs are extremely viscous and are resistant to heat and electricity. These qualities make them useful as lubricants and as insulators in electrical transformers (Osweiler *et al.*, 1985). The trade name assigned to technical PCB mixtures is Aroclor. This name is followed by a four-digit number, the first two digits of which signify the number of carbons present in the molecules and the last two digits of which signify the total percent chlorination of the mixture (Osweiler *et al.*, 1985). These compounds were manufactured from 1929 through 1976, when their further manufacture and use were banned. Over that 47-year time period, PCBs entered the environment through careless disposal at manufacturing plants or sites of use, accidents in electrical utilities or transformers, and accidental use in place of other chemicals.

Contamination of the Great Lakes Basin

In the 1960s, fur farmers using fish from the Great Lakes as a protein source in mink diets began observing increased adult mortality among their mink (Hartsough, 1965). Mink receiving lower amounts of Great Lakes fish in their diet were unable to reproduce. In addition to the effects on ranch-raised animals, populations of wild mink were apparently declining in the areas of the Great Lakes they had previously inhabited (Wren, 1991). As the extent of overtrapping, out-of-season extermination, and habitat destruction, and their effects on mink populations, are hard to assess, it cannot be stated with complete certainty that these decreases were not due to decreased habitat or food supply, or to overtrapping. However, research efforts directed at identifying the factor responsible for the increased mortality and reproductive problems suggested that contaminants such as PCBs were involved.

The effects of PCBs observed in fur-bearing animals and other wildlife led to a ban on new uses of PCBs, as well as their continued manufacture, in 1976. However, equipment such as electrical transformers which predated the new-use ban still contain PCBs are still in use. In some cases, improper removal or disposal of broken or outmoded equipment can lead to the entry of PCBs into the environment even 20 years after their ban. This entry has been estimated to be as much as eight million kgs each year (Feeley, 1995). As PCBs are only degraded slightly in the environment, the amounts of PCBs entering the environment currently, through inappropriate handling, are added to the amount released during the 47 years in which PCBs were used. The total amount of

PCBs in the lakes has declined since the mid-1970s due to both human and natural removal of PCBs from the environment (Tremblay and Gilman, 1995).

Bioaccumulation and Biomagnification of PCBs and PBBs

PCBs have a biphenyl structure with halogenation possible at up to 10 sites on the phenyl rings (Osweiler *et al.*, 1985). The hydrocarbon nature of the biphenyl skeleton makes PCBs very lipophilic, which allows them to partition into the fat stores of any organism consuming them. From the environment itself, PCBs are taken up by small organisms, such as plankton, at low trophic levels (Cordle *et al.*, 1978). Consumption of these PCB-containing organisms by animals in the next higher trophic level exposes these carnivores to an aggregate dose of PCBs. The PCBs are stored in the fat deposits of these animals, in the process of bioaccumulation, and the consumers in the next trophic level receive a higher dose of PCBs than did the animals they are consuming (Cordle *et al.*, 1978; Osweiler *et al.*, 1985). As this process of biomagnification continues through the food web, animals fairly high in the web (primary consumers) can be exposed to doses of these chemicals on the order of 5 mg PCBs/kg prey.

PCBs in Fish-Eater Cohorts

The organisms at risk of consuming toxic doses of HAHs include both humans and wildlife. A cohort of fish eaters (115 people) and regional controls (92 people) has been examined to determine whether fish consumption and lifestyle factors could be used to predict serum levels of lead, cadmium, PCB, and DDT (Hovinga *et al.*, 1993). Members of the fish-eater group were defined as those people who consumed 24 pounds

of sport-caught fish from the Great Lakes each year, while each person in the control group was from the Great Lakes region but ate 6 pounds per year or less of Great Lakes fish. Serum levels of DDT and PCBs in the fish-eater group were two and three times as high as in the control group, respectively. However, it was determined that long-term consumption of Great Lakes fish was a more important predictor of PCB and DDT contamination than was recent consumption of sport-caught fish (Hovinga *et al.*, 1993). As PCBs are trapped in sediments or removed from the ecosystem by removal of fish and other biomass, the PCB concentration in fish will decrease over time. Gradually, then, consumption of PCBs through consumption of Great Lakes fish will decrease (Tremblay and Gilman, 1995).

PCBs in Wildlife in the Great Lakes Basin

Wildlife is perhaps more exposed to dietary contaminants than are humans, because dietary modification is difficult at best in the natural environment. In heavily contaminated areas, wild animals may be exposed to concentrations of HAHs high enough to affect both their overall health and their ability to reproduce each year. A study conducted in New York State found that levels of PCBs and pesticides in wild-trapped mink and otter were correlated to the levels of those same compounds in fish from bodies of water within the ranges of the trapped animals (Foley *et al.*, 1988). PCB concentrations in adipose tissue samples from these trapped mink were measured to be as high as 95 ppm. The authors also reported that fish from PCB-contaminated areas such as the Hudson River watershed contain PCBs at concentrations greater than 0.64 ppm, which was shown to produce reproductive disruption in mink (Aulerich and Ringer,

1977). Although the LD50 of PCBs to mink differs with the extent and location of chlorination, the LD50s reported range from 0.05 ppm (dietary concentration) 3,4,5,3',4',5'-hexachlorobiphenyl over 135 days (Aulerich *et al.*, 1985) to 83 ppm (dietary concentration) Aroclor 1254 (Aulerich *et al*, 1986). The mink carcasses sampled in the New York State study were collected from trappers. Mink with acute exposure to PCBs or with extremely high tissue PCB concentrations would be very likely to die from PCB toxicity. Thus, the population represented in the New York State study is that group of mink exposed to low or intermediate concentrations of PCBs in the environment and their food supply. Despite this, approximately 10% of the carcasses they examined were found to have hepatic PCB concentrations higher than those observed in livers of ranch-raised mink that died while consuming 0.64 ppm PCBs in the diet. When wild mink were trapped in five Ontario townships near Lake Erie, PCB concentrations detected in whole-body homogenates ranged from 0.06 ppm up to 7.37 ppm. The mean PCB levels in mink from three townships (0.51 to 1.71 ppm) were higher than those reported in ranch-raised mink fed diets containing PCBs (Platonow and Karstad, 1973). The authors suggest that these PCB levels in the wild mink were high enough to cause reproductive disfunction (Proulx *et al.*, 1987).

Effects of PCBs in the Great Lakes Ecosystem

The concentration of PCBs and other compounds in the environment and in the animals in various parts of the ecosystem, and the potential for these contaminants to cause harm, has been assessed in a number of other studies throughout the Great Lakes basin as well as in other areas of the world. Cormorant eggs were collected from 11

colonies in the Great Lakes as well as from a reference colony in Manitoba, Canada. Extracts of the eggs were prepared to contain only PCBs (PCDDs and PCDFs were removed during the cleanup procedure) and added to culture medium of H4IIE rat hepatoma cell lines (Tillit *et al.*, 1992). The cytochrome P450IA1 inducibility of this cell line has been demonstrated previously to be a reliable indicator of dioxin-like activity (TCDD-equivalents) of polyhalogenated contaminants in biological material (Bradlaw and Casterline, 1979; Casterline *et al.*, 1983; Zacharewski *et al.*, 1989). The total PCB concentrations were also measured in the same samples (Tillit *et al.*, 1992). The hatching rate of eggs from the same nests as the eggs from which the PCBs were extracted was also monitored at 10 of the 11 Great Lakes sites and at the control site. Although the total PCB concentration in the eggs from the 11 Great Lakes colonies, as determined by GC analysis, was significantly correlated with egg mortality rates in the same colonies, the coefficient of determination was relatively low and was considered unreliable for predicting effects of PCB concentration. However, both the correlation of dioxin equivalents with egg mortality and the coefficient of determination were more significant than total PCB concentration. This suggests that the biological activity of polyhalogenated aromatic hydrocarbons may be a more reliable predictor of mortality in bird colonies in the Great Lakes than PCB concentrations themselves. (Proulx *et al.*, 1987).

Further analysis of the PCB fractions, and their biological activities, indicated that the cormorant egg extract was more active in the H4IIE bioassay than were technical mixtures such as Aroclor 1254. PCDDs and PCDFs were removed from the egg extract

during the extraction and cleanup process, thus the high biological activity of the egg extract is not due to these contaminants. The PCBs entering the environment, such as Aroclor 1254, therefore appear to be different from the chlorinated contaminants retained in fat stores of exposed animals. The increased biological activity in the H4IIE bioassay of the egg extract demonstrates the enrichment of environmental PCBs for those congeners which are more biologically active, and would explain why effects in both birds and other animals throughout the Great Lakes and other contaminated regions are still observed, while total PCB concentrations in the environment have decreased (Proulx *et al.*, 1987). After consumption, PCBs can be metabolized and excreted. The metabolism varies with the location of the chlorines on the biphenyl rings. PCBs with chlorines in the para positions are more rapidly metabolized and excreted, while congeners with chlorines at the ortho and meta positions are more likely to be retained in the tissues (Birnbaum, 1985; Borlakoglu and Haegele, 1991). Thus, although total PCB body burdens are decreasing, the PCBs left behind may be more biologically active than those that were consumed.

Cormorant chicks and small numbers of chicks from other species were collected from eight different areas throughout the Great Lakes region and examined for the incidence of bill malformation. The highest number of malformed chicks were found in the Green Bay, Wisconsin area. PCB congeners were isolated from the tissues of malformed chicks from cormorant colonies in that area (Stalling *et al.*, 1985). All seven species of birds in which abnormal development was observed in the Green Bay area are partially or totally piscivorous. The number of species affected and the common food

source suggested that the agent responsible for the malformations was in the diet. The authors suggested that finding developmental abnormalities in birds such as the cormorant in areas known to be contaminated will allow the use of the cormorant as a sentinel species for the developmental effects and overall biological activity of the contaminants in the Great Lakes basin (Fox *et al.*, 1991).

Concentrations of PCBs in seals throughout the Baltic Sea have been shown to range from 60-140 ppm in adipose tissue. PCB concentrations in herring, the main food source of the seals, from the same areas of the Baltic Sea were lower (5-25 ppm) than in the seals (Jensen *et al.*, 1979). Additionally, the patterns of PCBs observed in extracts from seals and from herring show that the PCBs retained by the seals had a higher general chlorination percentage than did the PCBs retained by the herring. The seal population was sampled during a subsequent breeding season to determine the number of pregnant, normal non-pregnant, and abnormal non-pregnant female seals, as well as to measure the concentration of DDT and PCBs in the same females. Nearly half (47%) of the seals were not pregnant and displayed abnormally narrowed or blocked uteri. In this same group, the mean PCB concentration was 110 mg/kg, while in the pregnant animals it was 73 mg/kg, and 89 mg/kg in the normal non-pregnant females. The difference between the PCB concentration in pregnant and abnormal non-pregnant female seals was significant (Jensen *et al.*, 1979). PCBs are able to cross the placenta, and this has been shown to occur in other animals, although to a much lower extent than across the mammary gland to the nursing young (Bleavins *et al.*, 1981). Transplacental movement of PCBs may well account for the lower PCB concentration in the pregnant female seals. It would be

beneficial to have measured PCB concentration (by sampling blubber through a recovery surgery) in a number of animals prior to the breeding season, and then to have correlated that to breeding success, as well as normality or abnormality of the uterus in each animal.

PCBS in Wild-trapped and Ranch Raised Mink

Although the population size of mink or other fur-bearing animals in a given area is usually based on reports from trappers rather than surveys by governmental agencies, using trapping results as a general guide to population size can be done carefully. Trapping returns from several areas of the North American continent have demonstrated declining numbers of mink over recent decades. In Ontario, for example, it is estimated that the highest level of wild mink harvested was about 60,00 mink per year between 1920 and 1988. The general trend over those 68 years has been a decline, to the 1988 level of 25-30,000 animals per year. Although this may be due to changing trapping methods that have reduced unintentional mink trapping, it is generally believed that the number of mink has declined in Ontario (Wren, 1991). This is reflected by the falling number of mink caught per trapper over four decades. When further comparisons were made over a time-span of 15 years between two highly PCB-contaminated areas and an area not contaminated with PCBs, lower numbers of mink were trapped in the highly contaminated areas than in the non-contaminated township (Jones and Glooshenko, 1990).

Data on mink harvests from Ohio indicate that the number of mink trapped in Ohio generally decreased from 1934 to approximately 1970, then began to increase again

(Wren, 1991). During that time, mink harvests (trapping, hunting, and other methods of collection) in Ohio counties bordering Lake Erie were lower than those counties farther inland (Wren, 1991). Although these data support the statement that PCB contamination of the environment, and thus of the mink, is the cause of the general decrease in mink populations in the Great Lakes basin, Wren (1991) suggests that caution is needed when interpreting the results. For example, residue levels must be measured in both wild mink and in the main components of their diet before the association of PCB contamination and decreasing populations can be considered reliable (Wren, 1991).

The adverse effect of PCBs on mink health was first identified in the 1960s, when fur farmers in the Midwest began including fish from the Great Lakes in the mink diet as an inexpensive protein source. When coho salmon from Lake Michigan were included at up to 15% of the diet fed to breeding stock, the breeding behavior of the animals was normal, but up to 80% of the kits born to dams fed diets containing Lake Michigan coho salmon died after birth (Aulerich *et al.*, 1973). The increased kit mortality appeared to be due to some contaminant in the diet of the dam, as the occurrence was related both to the percentage of the coho salmon in the diet and to the length of time the diet was consumed by the dams (Aulerich *et al.*, 1973).

A further study was designed and executed to determine whether the increased mortality was more likely due to rancidity of the diet, or to some contaminant in the diet. Mink were placed on diets containing ocean fish, coho salmon from Lake Michigan, Lake Erie, or the west coast, Lake Michigan bloater chub, or yellow perch from Lake Michigan

or Lake Erie. Ocean fish and west coast coho salmon were used for formulation of control diets to indicate whether the effects observed were due to some characteristic of the fish itself, or to a contaminant in the Great Lakes fish. In addition to examining the effect of including Great Lakes fish in the diet, part of the study examined the results of supplementing the diet containing ocean fish with 30 ppm PCBs. Finally, one group of mink was fed a diet containing ocean fish and supplemented with 10 ppm Aroclor 1254 and either 10 ppm DDT or 0.5 ppm dieldrin. This group was included to verify that the pesticide contamination of the Great Lakes fish used in other parts of the feeding study were not involved in the reproductive toxicity observed. Mink were bred within their dietary groups in all of these studies (Aulerich *et al.*, 1973).

A total of 47 female mink, fed diets containing either ocean fish or west coast coho salmon, were mated during the breeding season (Aulerich *et al.*, 1973). Of these mink, 44 whelped a total of 189 live kits (an additional 28 were stillborn), of which 153 were still alive at 4 weeks of age. In comparison, 65 female mink were fed diets containing 30% Lake Michigan coho salmon. The Lake Michigan salmon in these diets were shown to contain 10-15 ppm total PCBs, thus the dietary concentraton ranged from 3.3-5 ppm PCBs. Of these 65 females, only 6 carried a total of 10 kits to whelping, 8 of which were stillborn. The 2 live-born kits died within 24 hours. The mink whose ocean fish diets were supplemented with 30 ppm PCBs (Aroclors) did not whelp any kits. The results observed in mink fed diets containing other Great Lakes fish were less definitive; they ranged from no decrease in litter size or number of mink whelping (Lake Erie coho salmon, Lake Erie yellow perch, Lake Michigan yellow perch) to a 42% decrease in

number of females whelping, decreased litter size, and 55% still-born kits (Lake Michigan bloater chub). However, kit mortality by 4 weeks of age was increased in groups where dams were receiving yellow perch or bloater chub from Lake Michigan or coho salmon from Lake Erie when compared to the ocean fish or west coast coho salmon groups (Aulerich *et al.*, 1973).

In the groups receiving either Lake Michigan coho salmon or 30 ppm PCBs in the ocean fish diet, all of the adult mink died by the end of the whelping season. The clinical signs in the Lake Michigan coho salmon group were similar to those observed in the PCB-fed group (Aulerich *et al.*, 1973), suggesting that the mortality could be due to PCB-like contaminants in the salmon. Rancidity and mercury content of the fish were both ruled out as being the cause of mortality and decreased kit production. Although there was a relationship between litter size and total DDT or dieldrin contained in the Great Lakes fish, the dams did not demonstrate any evidence of toxicity from these pesticides. It was estimated that total pesticide consumption during the feeding trial would have been about 45 mg DDT (and metabolites) and 0.45 mg dieldrin. Previous studies in which DDT and DDT isomers were added at up to 150 ppm to the diet fed to mink for one year showed that reproductive performance was not affected by this pesticide (Aulerich and Ringer, 1970), which suggested that the much lower levels of DDT and dieldrin observed in the fish used here did not affect reproduction in the mink (Aulerich *et al.*, 1973). Tissues from the mink fed diets containing either Lake Michigan coho salmon or ocean fish supplemented with 30 ppm PCBs were analyzed for PCB residues. In control mink, PCB residues were measured to be less than 0.01 ppm. In both the Lake Michigan

salmon and the PCB-supplemented groups, however, PCB residues were much higher than in control animals. Residue concentrations in these two experimental groups were similar to each other, with the lowest concentrations found in heart (2.84 ppm, coho salmon group, 3.26 ppm, PCB-supplemented group) and the highest concentrations in brain tissue (11.07 ppm, coho salmon group, 11.00 ppm, PCB-supplemented group) (Aulerich *et al.*, 1973). The overall results, which include decreased reproductive success, increased adult mortality, similar clinical signs in adults, and similar PCB residue concentrations, in groups fed either PCBs or Lake Michigan coho salmon, strongly suggested that PCBs were involved in the reproductive problems observed in mink fed Great Lakes fish (Aulerich *et al.*, 1973).

In another feeding study, designed to examine the toxicity of technical mixtures of PCBs to mink was examined in another feeding study, adult mink were placed on diets supplemented with up to 40 ppm Aroclor 1242 or 20 ppm Aroclor 1016. These two compounds differ only slightly in the amount of chlorine they contain, although Aroclor 1242 contains a higher percentage of highly chlorinated molecules than does Aroclor 1016. Additionally, Aroclor 1016 contains a lower percentage of chlorinated dibenzofurans (PCDFs) and dibenzodioxins (PCDDs) than Aroclor 1242. The toxicity of these two mixtures, as well as other HAHs, could be mediated either through the percentage and site of chlorination, or by the contamination of the Aroclors by PCDFs and PCDDs. Adding either Aroclor 1016 or Aroclor 1242 to the diet of breeding mink allows the preliminary investigation of which characteristic is involved in Aroclor toxicity. If Aroclor 1016 is less toxic to mink than Aroclor 1242, then the high

chlorination per molecule, and the contamination of the mixture with PCDDs and PCDFs, is more important than the total percentage of chlorines in the molecule. However, the relative importance of high molecular chlorination and PCDD or PCDF contamination cannot be separated in an experiment such as the one described here.

Lethality of Aroclor 1242 to adult mink was higher than that of Aroclor 1016, and females were more sensitive than males to either of the Aroclor mixtures. Consumption of Aroclor 1242 for four months prior to the breeding season led to complete reproductive failure in that no kits were whelped, although mating behavior appeared to be normal. These effects were observed even at 5 ppm Aroclor 1242, the lowest dose used in this study. Adding Aroclor 1016 to the diet at 20 ppm decreased but did not abolish reproduction; 4 of the 9 females mated whelped kits even after four months consumption of Aroclor 1016. The percentage of live kits was not reduced in the mink that did whelp while consuming diets containing Aroclor 1016. However, weight gain in the kits by four weeks of age, among kits born to dams consuming Aroclor 1016, was decreased compared to kits in control litters. Mortality in these Aroclor 1016 litters between birth and four weeks was also higher than in control litters (Bleavins *et al.*, 1980).

A short-term study in mink examined the gestational and lactational transfer of PCBs from dam to kit (Bleavins *et al.*, 1981). The results of that experiment demonstrated that while there is some transmission of PCBs from dam to kit during gestation, most transfer occurs during lactation. Up to 16 times more PCBs were taken up by the kits between birth and two weeks of age than during the last stage of gestation (Bleavins *et al.*, 1981). Lactational transfer would explain the increased kit mortality by

four weeks of age in the groups of mink kits whose dams were fed diets supplemented with Aroclor 1016 (Bleavins *et al.*, 1980), and would suggest that kits are more susceptible to the toxicity of HAHs than the adults. Furthermore, the greater effects observed in the mink treated with Aroclor 1242 than in the mink consuming Aroclor 1016 suggests that reproductive toxicity and adult mortality both are due either to the higher percentage of highly chlorinated molecules in Aroclor 1242, or to the higher contamination of Aroclor 1242 with PCDFs and PCDDs.

The efficiency of lactational transfer of PCBs to the kit was also demonstrated in a study examining the effect of long-term Aroclor 1254 consumption in mink. Adult mink were fed diets containing 1.0 ppm Aroclor 1254 starting approximately three months before breeding, through weaning of the kits. This resulted in adult consumption of PCBs over a 7 month time span. Additional mink were sacrificed at approximately 2, 4, and 6 months after the start of the feeding study, and hepatic PCB concentrations were measured. Kits were sacrificed at 5 weeks of age, about the time that they would begin eating solid food, and hepatic PCB concentrations were measured. In 5-week-old kits, hepatic PCB concentration was near that observed in adult mink that had consumed PCB-containing diets for 4 months (Wren *et al.*, 1987a), further supporting the role of lactation in the transfer of PCBs from dams to kits.

A later experiment examined the effect of long-term feeding of Great Lakes fish to both kit and adult mink (Hornshaw *et al.*, 1983). Whole fish, fish trimmings or fishmeal from five different species of Great Lakes fish were added to the diet to provide

PCB concentrations from 0.21 to 1.50 ppm. Female mink fed carp (1.50 ppm PCBs in the final diet) did not whelp any live kits, although three of the nine females mated whelped a total of 9 stillborn kits. Reproduction was not affected in any of the other dietary groups. In the second part of the experiment, female mink kits were placed on diets containing 0.66 ppm PCBs through the inclusion of both Lake Erie perch scraps and Saginaw Bay (Lake Huron) sucker scraps in the diet. Kits were fed these diets beginning at the age of 8-10 weeks, and continuing through breeding, gestation, and lactation, for approximately 11 months. These female mink were bred to non-treated males. When the results from these mink were compared to those for animals fed diets containing ocean fish, significant reductions were observed in the number of kits whelped, average number of live kits whelped per dam, and number of kits still alive at 4 weeks of age (Hornshaw *et al.*, 1983). These results are similar to those collected in studies in which both male and female mink were fed diets containing Great Lakes fish. As the perch and sucker, and thus the PCBs were fed only to the females in this study, the results strongly suggest that PCBs affect female reproduction. The authors calculated that the female mink fed perch scraps and sucker for 10-11 months, from 8 weeks of age through whelping, consumed approximately 29 mg PCBs (Hornshaw *et al.*, 1983). When a technical mixture of PCBs (Aroclor 1254) was added to the diet at 2 ppm for 9 months in another experiment, decreased reproduction was observed (Aulerich and Ringer, 1977). This level of Aroclor 1254 was the lowest at which any effect on reproduction was observed. Following the same method of calculation, the mink fed Aroclor 1254 at 2 ppm consumed approximately 81 mg PCBs. Thus, "metabolized" PCBs produce reproductive toxicity at a concentration which is one-third the level of non-metabolized, technical

material required to produce the same effect, based on dietary PCB concentration and the total amount of PCBs consumed.

The increased toxicity of metabolized PCBs was supported by a later experiment in which mink were either fed carcasses of rabbits previously treated with Aroclor 1254 (secondary toxicity from metabolized PCBs) or were themselves administerd Aroclor 1254 through the diet (primary toxicity) (Aulerich *et al.*, 1986). In both groups, the dietary PCB concentrations ranged from 7 to 75 ppm. The LC_{50} (concentration lethal to 50% of the population) was found to be lower in the mink fed metabolized Aroclors (47.0 ppm at 28 days, 31.5 ppm at 35 days) than in the group consuming non-metabolized Aroclors (79.0 ppm at 28 days, 48.5 ppm at 35 days) (Aulerich *et al.*, 1986). It has been suggested that metabolism of Aroclor enriches the mixture for the more highly chlorinated PCB congeners, which are generally the more toxic congeners as well as being more resistant to metabolism than the less-chlorinated PCB congeners (Matthews *et al.*, 1978; Hornshaw *et al.*, 1983). This would help to explain why the use of fish in the diet causes reproductive failure at a lower PCB concentration than the addition of technical PCB mixtures to the mink diet (Hornshaw *et al.*, 1983).

The addition of 2.5 ppm Aroclor 1254 to the diet of female mink produced almost total reproductive failure, with only one stillborn kit whelped (10 female mink were mated to untreated males). Mink fed either 2,4,5,2',4',5'-hexachloro-biphenyl (245-HCB) or 2,3,6,2'3',6'-hexachlorobiphenyl (236-HCB) showed no signs of reproductive toxicity at dietary PCB concentrations of 2.5 or 5.0 ppm. Other groups of mink were fed

3,4,5,3',4',5'-hexachlorobiphenyl (345-HCB) at 0.1 or 0.5 ppm in the diet, and had 100% adult mortality with no kits whelped (Aulerich *et al.*, 1985). These concentrations of 345-HCB in the diet resulted in the consumption of 0.9 mg (0.1 ppm in the diet) or 3.1 mg (0.5 ppm in the diet), demonstrating the extreme toxicity of this congener in mink. In the mink fed 345-, 236-, or 245-HCB, or Aroclor 1254, there were no changes in 17ß-estradiol concentrations in the blood. Although both Aroclor 1254 and 345-HCB produced similar effects on reproductive success, these two PCBs had opposite effects on plasma progesterone concentrations (Aulerich *et al.*, 1985). This suggests that alteration of steroid concentrations may not be involved in reproductive decreases by PCBs. In mink fed diets containing 1.0 ppm Aroclor 1254, there was no decrease in the number of females whelping kits or in the number of kits whelped per dam. However, kit growth rate during lactation was decreased in this dietary group when compared to control kits (Wren *et al.*, 1987b).

In a more complex study, PCB mixtures were fractionated into sub-components containing different percentages of ortho chlorination. The reproductive toxicity of fractions with different numbers of chlorines ortho to the biphenyl bond, as well as the non-PCB contaminants from the technical mixture, was then examined. The PCB fractions and technical mixtures were added to the diet to be equal to 2 mg Clophen A50 or 1.64 mg Aroclor 1254 per day. Additional groups received either 2 mg/day Clophen A50 or 1.64 mg/day Aroclor 1254. A significant reduction in pregnancies carried to term was observed in the groups fed diets containing either Clophen A50 or Aroclor 1254 (Kihlstrom *et al.*, 1992). Reduced pregnancies were also observed in groups fed

combinations of Aroclor 1254 fractions that contained at least 1 ortho substitution. PCB concentration was also increased in the dams fed these diets (Kihlstrom *et al.*, 1992).

When carp were fed to mink to provide up to 2.56 mg PCBs/kg diet prior to and during breeding, gestation, and lactation, decreased reproductive performance was observed. In the group receiving 2.56 mg/kg PCBs (through the addition of 40% carp to the diet), the number of live kits whelped per female decreased, the number of stillborn kits increased, and the average litter size also decreased. All changes were significant when compared to the control group. Kits born to dams on the 40% carp diet did not survive through three weeks of age (Heaton *et al.*, 1995). This decrease in kit survival indicates that PCBs consumed by the dam are transferred through the milk to the kits and are toxic in the kits.

The major points to be developed from these studies are that 1) the effect of PCBs on mink reproductive potential appears to be limited to the female; and 2) given the variety of effects observed (such as anestrus, decreased litter size, fetal resorption), it is likely that PCBs are affecting some controller of reproduction that is high in the reproductive and endocrine heirarchy. Estrogen and the ability of normally estrogen-responsive tissues to detect estrogen are candidates for the cause of disruption. Increasing estrogen concentrations cause ovulation, while estrogen up-regulates the progesterone receptor in the uterus. This hormone is essential for the maintenance of normal pregnancies. The effects of PCBs and similar compounds on the estrogen/estrogen

receptor system have been examined in attempts to determine how PCBs affect reproduction.

Section 3

The Anti-Reproductive Mechanism of PCBs

The potential for PCBs to decrease reproductive ability appears to be mediated through an antiestrogenic effect. There are two hypotheses to explain the potential mechanism(s) by which these compounds act in an antiestrogenic manner.

Degradation of Estradiol

The first hypothesis suggests that the induction of hepatic cytochrome P450IA1 by the PCBs increases the degradation of estradiol in the body, and that circulating estradiol concentrations then fall below their effective threshold for regulating the reproductive cycle. In this scenario, the signal responsible for coordinating gene transcription, protein synthesis, tissue development, and other reproductive events is not sent from the ovaries to the other estrogen-responsive tissues in the body (uterus, hypothalamus). The hypothesis of increased estradiol degradation is based primarily on observations made in estrogen-responsive, HAH-responsive MCF-7 cells. When these cells were treated with 2,3,7,8-TCDD, the concentrations of estradiol metabolites increased, and the concentration in the culture medium of estradiol decreased (Spink *et al.*, 1990). TCDD (10 nM) was added to the medium of confluent cell cultures and the medium was left on the cells for 72 hours. Following this pre-treatment period (to allow induction of cytochrome P450 enzymes), the culture medium was switched to one containing 500 nM

estradiol. After a four hour incubation period, the medium was collected and analyzed both for estrogens and for hydroxylated estrogen metabolites (Spink *et al.*, 1990). Additionally, a portion of the culture medium was treated with ß-glucuronidase and sulfatase to determine the extent of conjugation of the estrogen metabolites. The results of this study indicate that hydroxylated metabolites were increased in cultures pre-treated with TCDD for 72 hours prior to the addition of estradiol to the medium (Spink *et al.*, 1990).

These authors conducted a similar study, again in MCF-7 cells, showing that in cells pre-treated with 10 nM TCDD, there is a significant induction of cytochrome P450IA1 and cytochrome P450IA2 activity. Medium from TCDD-pre-treated MCF-7 cells was shown to have a decreased estrogen concentration and increased concentrations of the hydroxylated and methoxylated metabolites of estrogen (Spink *et al.*, 1991). An additional experiment examined the production of estrogen metabolites when estrogen was incubated with microsomes from untreated or TCDD-pre-treated cells. Enzyme activity in the microsomes from the TCDD-treated cells produced four metabolites of estrogen that had not been detectable after incubation of estrogen with microsomes from untreated cells (Spink *et al.*, 1991). The authors suggest, based on the results of these two studies, that increased degradation of estradiol in individuals exposed to AhR ligands such as TCDD may be one way in which TCDD exerts its antiestrogenic effects. Increased concentration of an estrogen metabolite apparently produced by the action of cytochromes P450IA1 and P450IA2 has been observed in tissues from human female smokers (Michnovicz *et al.*, 1986).

The ability of AhR ligands such as TCDD to decrease estradiol concentrations and increase concentrations of estrogen metabolites in the medium of cultured cells is clear. The significance and applicability of these results to the whole animal condition is less obvious. In the whole animal, estrogen is produced at the ovary by enzymes whose activity is increased by follicle-stimulating hormone (FSH), from the adenohypophysis. The production of increasing amounts of estrogen then stimulates the surge in release of both FSH and luteinizing hormone (LH, also from the adenohypophysis) to cause ovulation (Randall *et al.*, 1997). These hormone cycles are also under the regulation of other hormones at the level of both the adenohypophysis and the ovary itself. Given the intricate interrelationships of hormones throughout the body and the body's tendency towards homeostatic compensation, it is possible that if circulating estrogens decrease, the ovarian production of estrogens may increase in compensation, and thus the concentration of circulating estradiol would not change.

Downregulation of the Estrogen Receptor

The second hypothesis is that the antiestrogenic action of AhR ligands is mediated through effects on estrogen receptor function, rather than alterations in circulating estrogen concentration. In a model proposed by Safe *et al.* (1991), there are several suggested ways in which AhR ligands such as TCDD could act as antiestrogens. The mechanism which has the most experimental evidence to support it is that the AhR-ligand complex could directly inhibit transcription of genes whose transcription is ordinarily induced by estrogen.

The results of several studies suggests that this mechanism at least in part explains the antiestrogenic effect of TCDD and other AhR ligands. TCDD administered to mice produced a decrease in mRNA for the estrogen receptor (White and Gasiewicz, 1993), one of the proteins whose synthesis is increased by estradiol. The observed decrease in ER mRNA may have been due to decreased stability of the estrogen receptor mRNA as well as decreased transcription (White and Gasiewicz, 1993). Estrogen administered to sexually immature (25-day-old) female Sprague-Dawley rats increased uterine levels of c-fos mRNA (Astroff *et al.*, 1991). When these animals were treated with either of two AhR ligands, the constitutive expression of the c-fos gene decreased significantly compared to control. Administering both estradiol and the AhR ligand decreased the estrogen-induced transcription of the c-fos gene when compared to estradiol alone (Astroff *et al.*, 1991). The transcription of c-fos in animals treated with both estradiol and TCDD only fell below that observed in non-treated animals at the last time-point examined, 48 hours after treatment with both compounds (Astroff *et al.*, 1991). In uteri of rats treated with estradiol, expression of epidermal growth factor receptor (EGFR) mRNA increases. When immature female rats were treated with TCDD, the constitutive expression of EGFR mRNA decreased, as did c-fos mRNA expression(Astroff *et al.*, 1990). As with the expression of c-fos, co-treatment with both estradiol and TCDD inhibited the estradiol-induced increase in EGFR mRNA expression, and actually decreased EGF expression below that observed in control animals (Astroff *et al.*, 1990).

The expression and level of marker proteins could also be used to examine the mechanism by which TCDD and other, similar compounds exert their antiestrogenic effects. One such protein that could be used is pS2. This small protein, apparently a signalling peptide, is released by MCF-7 cells into the surrounding medium. Nuclear run-off assays demonstrated that the induction of this gene by estradiol occured at the level of transcription, and was unaffected by inhibition of protein synthesis (Brown *et al.*, 1984). The induction of transcription of pS2 by estradiol is evident within 15 minutes of estradiol addition to the culture medium, indicating that pS2 transcription is a primary effect of estrogen receptor binding to DNA. Co-treatment of cells with both estradiol and TCDD significantly inhibited the expression of pS2 normally stimulated by estradiol. Mutant Hepa1c1c7 cells that possess a functional AhR but do not show induction of cytochrome P450IA1 in response to TCDD treatment were used to examine the ability of TCDD to down-regulate pS2 expression. In this cell line, the inhibition by TCDD of the estrogen-mediated pS2 induction was similar to that observed in the wild-type cells (Zacharewski *et al.*, 1994). If induction of cytochrome P450IA1 were necessary for the antiestrogenic effects of TCDD and other AhR ligands, then this result would not be expected. Two other mutant cell lines were examined for their response to estradiol and TCDD co-treatment. One cell line is deficient in Ah Receptor Nuclear Translocating Protein (ARNT), while the other cell line lacks the AhR. Administration of estradiol in these cell lines greatly induced the expression of pS2. However, the addition of TCDD with estradiol had no effect unless the appropriate protein was transfected into the cell line prior to co-treatment with estradiol and TCDD (Zacharewski, *et al.*, 1994).

This inhibition of estrogen-mediated transcriptional induction might occur through direct physical blockage of the transcriptional machinery on the DNA strand. The human ER gene has been shown to contain a number of sequences identical to those, commonly called dioxin response elements (DREs), to which the AhR binds. The first exon of the human ER contains one full-length and one partial DRE. In a standard gel retardation assay, a radiolabeled oligonucleotide from this region was incubated with nuclear extracts from either untreated or TCDD-treated Hepa1c1c7 cells, prior to non-denaturing gel electrophoresis (White and Gasiewicz, 1993). For a positive control, an oligonucleotide containing the DRE from the TCDD-inducible cytochrome P450IA1 gene was also incubated with nuclear extracts from TCDD-treated or untreated cells. On the resulting autoradiogram, retardation of the radiolabeled, DRE-containing oligonucleotide from both the human ER gene and the murine cytochrome P450IA1 gene was consistent with binding of the liganded AhR from the nuclear extracts of TCDD-treated cells. However, it would be interesting to incubate the human ER oligonucleotide with a nuclear extract from TCDD-treated class 1 mutant (AhR-deficient) Hepa1c1c7 cells, in order to demonstrate that the nuclear extract constituent binding to the human ER oligonucleotide is indeed the liganded AhR, rather than some other, unidentified protein.

The intensity of the shifted band observed with incubations of the nuclear extract from TCDD-treated Hepa1c1c7 cells depended on the TCDD concentration administered to the cultured cells prior to nuclear extract preparation. Additionally, the band did not appear when extracts from untreated cells were used (White and Gasiewicz, 1993),

suggesting that the liganded AhR was indeed the nuclear extract component binding to the DRE.

The unlabeled human ER DRE-containing oligonucleotide was able to compete with the labeled murine cytochrome P450IA1 oligonucleotide, although the IC_{50} (50% inhibitory concentration) for the human oligonucleotide was higher than for the corresponding unlabeled mouse oligonucleotide. When a point-mutated human ER DRE-containing oligonucleotide was used, even at a 400-fold excess, the mutant oligonucleotide was unable to abolish binding of radiolabeled mouse oligonucleotide to the nuclear extract. Use of the non-mutated human oligonucleotide at the same 400-fold excess concentration completely competed for binding of the nuclear extract from TCDD-treated cells. These results indicate that the DRE in exon I of the human ER gene is, at least *in vitro*, capable of binding liganded AhR. In addition to this DRE, the human ER gene has five full or partial DREs upstream of the gene, in the regulatory region. The binding of the AhR to one or more of the DREs in the human ER gene could inhibit expression of ER in response to estradiol administration by 1) blocking RNA polymerase activity (as in the case of the DRE in exon I); or 2) inhibiting the binding of other transcription factors to the ER regulatory region by blocking or overlapping their binding sites and thus preventing transcriptional activation (White and Gasiewicz, 1993).

Other mechanisms suggested by Safe *et al.* (1991) include the synthesis of as yet-unidentified modulatory proteins induced by the AhR would inhibit estrogen receptor-mediated induction of transcription of estrogen-responsive genes or would act as antimitogens. The evidence for these proposed mechanisms by which AhR ligands may

act as antiestrogens is somewhat equivocal. In Hepa1c1c7 mouse hepatoma cells treated with estradiol, nuclear estrogen receptor concentrations increased. When these cells were pre-treated with TCDD and then stimulated with estradiol, the estrogen-induced increase in nuclear ER was inhibited (Zacharewski *et al.*, 1991). If the cells were treated with either actinomycin D (an inhibitor of transcription) or cycloheximide (a protein synthesis inhibitor), TCDD did not inhibit the estrogen-induced increase in nuclear ER concentration (Zacharewski, *et al.*, 1991). Similar results were observed when 6-methyl-1,3,8-trichlorodibenzofuran (6-MCDF), an AhR ligand, was added to the culture medium of MCF-7 cells. If 6-MCDF was added to the culture medium prior to estradiol, the concentration of ER detectable either by receptor binding assays or by immunodetection methods was not increased by estradiol. Treatment of the cells with either actinomycin D or cycloheximide prevented the inhibitory effect of 6-MCDF (Zacharewski, *et al.*, 1992). This experiment suggests that the effect of AhR ligands on estrogen receptor concentration is mediated at least in part through the TCDD-induced synthesis of some intermediate protein.

Progesterone was shown to antagonize the estrogen-mediated induction of both uterine estrogen receptor and uterine progesterone receptor in rats (Romkes and Safe, 1988). As with the AhR ligands used previously *in vitro*, in freshly isolated rat uterine strips this effect could be blocked by the administration of either actinomycin D or cycloheximide. Experiments from the same lab group showed, however, that in ex vivo conditions (rat uterine strip assays), the ability of TCDD incubated with the uterine tissue to antagonize an estrogen-induced increase in ER and PR could be blocked by

actinomycin D, but not by cycloheximide (Romkes and Safe, 1988, 1989). These results suggest that progesterone and TCDD are both antiestrogenic, but that they act to decrease estrogen receptor concentrations through different mechanisms. Further, the comparison of *in vitro* (Zacharewski, *et al.*, 1991, 1992) and ex vivo results with regard to the anti-estrogenic mechanism of TCDD suggests that there is most likely more than one pathway by which AhR ligands are antiestrogenic.

This multiplicity of mechanisms for AhR ligands is further borne out by the effect of 6-nitro-1,3,8-trichlorodibenzofuran (6-NCBF). Although this compound binds to the AhR and could thus be expected to exhibit antiestrogenic effects, administration of 6-NCBF to rats increased uterine wet weight. Co-administration of both 6-NCDF and estradiol increased uterine wet weight to the same extent as estradiol alone. While estrogen increased uterine peroxidase activity and EGF receptor concentration in the uterus, 6-NCBF inhibited uterine peroxidase activity (thereby acting as an antiestrogen) and had no effect on EGF receptor concentration (Dickerson *et al.*, 1992). Thus, one AhR ligand can act as both an antiestrogen and an estrogen.

In the whole animal, the effect of HAHs such as PCBs and PCDDs appears to be mediated through the AhR. Several different AhR ligands, including some with high affinity and some with low affinity for the receptor, were examined in the rat for their effect on uterine estrogen receptor. In general, the compounds with lower affinity for the AhR needed a higher dose to produce the same effect as a low dose of a high-affinity compound (Romkes *et al.*, 1987). For example, 2,3,7,8-TCDD decreased hepatic ER

concentrations by 42% when administered at a level of 80 μg/kg. A lower-affinity AhR ligand, 1,3,7,8-TCDD, decreased hepatic estrogen receptor by 40% only when given at 400 μg/kg (Romkes *et al.*, 1987). Similar relationships between affinity for the AhR and potency as antiestrogens were observed when the effects of 2,3,7,8-TCDD and 1,2,4,7,8-pentachlorodibenzo-*p*-dioxin (PeCDD) on uterine peroxidase and uterine wet weight were compared in the rat (Astroff and Safe, 1990). The probable involvement of the AhR receptor in the antiestrogenic effect of HAHs was further demonstrated by the results of treating two related strains of mice with TCDD. One strain, the DBA/2 mouse, is classified as non-responsive and is not susceptible to the overall health effects of AhR ligands except at very high doses. The other strain, the C57Bl/6 mouse, differs by only one gene from the DBA/2 strain and is Ah-responsive (Thomas *et al.*, 1972). In the C57Bl/6 mouse, TCDD decreased hepatic estrogen receptor concentration by 30% when administered in a single dose at 30 μg/kg (Lin *et al.*, 1991). Although the DBA/2 mouse was not treated with TCDD, a non-Ah-responsive variant of the C57Bl/6 mouse treated with TCDD did not demonstrate a decrease in hepatic estrogen receptor concentration (Lin *et al.*, 1991).

The down-regulation of estrogen receptor by AhR ligands exhibits tissue-specific characteristics. In CD1 mice administered TCDD at up to 30 μg/kg, both hepatic and uterine ER were decreased. While hepatic ER concentrations remained low for 21 days after TCDD treatment, uterine estrogen receptor concentration rebounded within 14 days. This was the same time frame as the recovery in uterine wet weight, suggesting that

decreased ER mediates the antiestrogenic effects of TCDD, and that the increasing uterine ER concentration allowed uterine weight to increase as well (DeVito *et al.*, 1992).

The general trends demonstrated by these studies are that the affinity of a HAH for the AhR is related to the antiestrogenic activity of that compound both in the whole animal and *in vitro*, and that the effect observed of treatment with an AhR ligand may well depend on the tissue examined.

The possibility for increased degradation of estradiol to be one effector of antiestrogenicity of AhR ligands loses support when the results of studies both *in vitro* and in the whole animal are considered. Rats were treated with either TCDD or 6-MCDF, and the effects on hepatic cytochrome P450 activity and hepatic and uterine estrogen receptor concentrations were determined. In addition, the relative potencies of these two chemicals for cytochrome P450 induction and estrogen receptor decrease were calculated. TCDD was demonstrated to be 1.5×10^5 times more potent than 6-MCDF when considering the induction of cytochrome P450 enzymes. However, TCDD was only 700 times more potent than 6-MCDF in terms of decreasing estrogen receptor concentration. Additionally, the down-regulation of estrogen receptor in rats treated with 6-MCDF occurred with only a minimal induction in cytochrome P450, suggesting that decreased estrogen concentrations are probably not involved in the AhR-mediated antiestrogenic effect (Astroff and Safe, 1988). In CD1 mice, one administration of TCDD at 30 μg/kg increased cytochrome P450 concentration and activity 2 days after treatment.

Serum estradiol did not change throughout the duration of the experiment. These results were consistent throughout the 21 days of the experiment (DeVito *et al.*, 1992).

In Hepa1c1c7 cells, the effect of TCDD on estrogen receptor concentrations can be observed as early as 1-1.5 hours after treatment, which is too early for any but the most minimal induction of cytochrome P450 enzymes (Zacharewski *et al.*, 1991). This further supports the suggestion that the decrease in estrogen receptor concentration observed after administration of or exposure to AhR ligands is probably not due to the cytochrome P450-mediated degradation of estradiol.

Section 4

Potential for a Biomarker for PCB Exposure

Cytochrome P450 Activity and Induction

In addition to decreases in reproductive ability, PCBs are also able to cause other alterations in animal physiology and biochemistry. One of the more easily measured changes is the induction of the hepatic cytochrome P450 enzyme system. This enzyme family is made up of several hundred different isozymes, which occur in both plants and animals. The separate enzymes are classified and named according to their amino acid (and/or nucleotide) sequence. The overall group, P450, is divided into superfamilies which are denoted by Roman numerals after the superfamily name. Enzymes in the same superfamily share more than 50% but less than 95% sequence identity with each other. The superfamily is then subdivided into families given the same capital letter after the Roman numeral; members within families have at least 95% sequence identity. Finally,

the separate enzymes within the family are assigned Arabic numerals to identify the different isozymes. Thus, cytochrome P450IA1 and cytochrome P450IA2 have at least 95% sequence similarity to each other, but only 50% or less sequence identity with cytochrome P450IIB1. The names also cross species boundaries, ensuring that cytochrome P450IA1 in mice is the same as cytochrome P450IA1 in mink, rats, Drosophila, or any other organism. Since their initial discovery and characterization in 1964, the number of identified cytochrome P450 enzymes has increased every year.

Discovery and Isolation of Cytochrome P450

The hepatic xenobiotic-metabolizing enzyme system first received attention in the mid-1950s. It was demonstrated that the diet, as well as particular components of the diet such as protein or carbohydrate content, vitamins A and C, and lipid content, could affect the enzyme activity observed in liver homogenates (Brown *et al.*, 1954; Butler and Dauterman, 1988; Yoo *et al.*, 1990; Yang *et al.*, 1992).

It was then shown that administration of 3-methylcholanthrene (3-MC), a polycyclic aromatic hydrocarbon, to rats could increase the N-demethylation of aminoazo dyes (Conney *et al.*, 1956). The *in vitro* addition of other inducers of enzyme activity to liver homogenates did not increase N-demethylase activity (Conney and Burns, 1959; Conney *et al.*, 1956; Conney *et al.*, 1957; Conney and Burns, 1963), and the in vitro addition of protein synthesis inhibitors did not decrease this activity. The administration of a protein synthesis inhibitor to an animal simultaneously with 3-MC, however, decreased the N-demethylase activity of the homogenated prepared from the livers of

these treated animals (Conney and Burns, 1959; Conney *et al.*, 1957; Conney and Burns, 1963). This, combined with studies in which the incorporation of ^{14}C-leucine into liver protein increased after treatment of rats with phenobarbital, a known inducer of N-demethylase activity (Kato *et al.*, 1965), confirmed that protein synthesis was increased by inducers of enzyme activity.

Liver homogenates which were reduced with sodium dithionite and treated with carbon monoxide were shown to have a spectral absorption peak at 450 nm (Klingenberg, 1958; Omura and Sato, 1964). The protein responsible for this peak was identified as a cytochrome protein functioning in electron transport, and was thus named cytochrome P450.

Induction Mechanisms

Cytochrome P450 enzymes are inducible, meaning that their activity can be induced by administration of various chemicals. Often, this induction occurs through increased transcription of the messenger RNA (mRNA) for the protein, and transcription of this increased message to produce more of the enzyme. Thus, although the maximal velocity of each enzyme molecule may not have changed, the metabolism of substrate to product has increased because there is more enzyme available. The induction of cytochrome P450IA enzymes by HAHs has been well-characterized in a number of reports (Harada *et al.*, 1981; Guengerich, 1978). It has been further shown that the binding of the specific HAH to the AhR in the cell is correlated to the ability of that HAH to induce hepatic cytochrome P450IA1 enzymes; hydrocarbons to which the AhR

demonstrates a high binding affinity are very good inducers, while those which are bound only weakly by the receptor do not induce well. Induction of cytochrome P450IA by HAHs has also demonstrated a dose-response relationship, where the activity of cytochrome P450IA increases as the dose of HAHs is increased.

The induction of cytochrome P450 activity can be measured relatively easily with *in vitro* techniques. Homogenization and ultracentrifugation of the hepatic tissue yield a preparation highly enriched for the rough endoplasmic reticulum, the segment of the cell that contains the cytochrome P450 enzymes. This fraction contains vesicles, or microsomes, of the enzyme-containing endoplasmic reticulum. The enzymes must be provided with electrons to carry out their reactions, thus an electron-generating system (usually containing glucose-6-phosphate, glucose-6-phosphate dehydrogenase, and NADP) is added to the microsomes when measuring cytochrome P450 activity. The activity of cytochrome P450 enzymes toward various substrates has been well-characterized, and the metabolism of different substrates has been reliably assigned to specific cytochrome P450 isozymes (Bock *et al.*, 1990). Many of the substrates are chemicals whose products from cytochrome P450-mediated reactions are either fluorescent or are easily detected by spectrophotometric means (Burke *et al.*, 1985; Lubet *et al.*, 1985). In addition to the development of many *in vitro* assays for cytochrome P450 activity and induction, investigators are beginning to develop *in vivo*, non-invasive methods for measuring cytochrome P450 activity in live-trapped animals.

Cytochrome P450 as an Indicator of PCB Consumption

If non-invasive biomarkers such as cytochrome P450 activity can be developed to indicate exposure to contaminants such as PCBs, the impact of contaminated environments on the health of animals living or being released in them can be more readily assessed. One such method of measuring hepatic cytochrome P450 enzymatic activity involves the use of caffeine as a substrate. Caffeine is metabolized by several cytochrome P450 enzymes, including cytochrome P450IA1 and P450IA2, and the metabolites can be detected in urine (Chung *et al.*, 1998) Recent experiments have shown that substrates for several human cytochrome P450 isozymes can be combined into one bolus, and the urine analyzed for metabolites (Frye *et al.*, 1997). Trapping wild mink and administering either unlabelled or radiolabelled substrates, collecting urine for metabolite analysis, and then releasing the animal would allow the determination of hepatic cytochrome P450IA enzyme activity without decreasing the number of animals in the population.

Experiments such as those described in this dissertation may provide a baseline for further research into predictions of environmental health and animal reproductive fates in the environments. For example, one avenue of further experimentation should focus on development of non-invasive methods for measuring hepatic cytochrome P450 activity in mink. Other research should include a clear determination of the antiestrogenic mechanism of PCBs in mink. Once the mechanism of reproductive toxicity of PCBs in mink is clear, and the dose-response relationship of PCBs and reproductive disfunction has been elucidated, correlation of reproductive endpoints with

non-invasive cytochrome P450 activity measurements can be examined. This information could then be applied to field studies to determine whether or not hepatic cytochrome P450 induction can be used as a predictor of reproductive capability in the mink.

Chapter 2

LIVER PCB CONCENTRATION AND INDUCTION OF HEPATIC CYTOCHROME P450 ACTIVITY AS A POTENTIAL BIOMARKER FOR PCB CONSUMPTION

INTRODUCTION

The concentration of industries around the Great Lakes basin has resulted in the contamination of the ecosystem with a variety of organic chemicals. Among these compounds are the halogenated aromatic hydrocarbons (HAHs), which include polychlorinated biphenyls (PCBs), polychlorinated dibenzofurans (PCDFs), and polychlorinated dibenzo-p-dioxins (PCDDs). These compounds are highly lipid-soluble and readily partition into fat tissues of animals consuming them. With this storage process occurring at each level of an ecosystem, compounds such as PCBs will be both bioaccumulated (stored and passed to the next highest level of the ecosystem) and biomagnified or bioconcentrated (passed on in higher concentrations at each trophic level) (Borlakoglu and Haegele, 1991). Additionally, most HAHs are stable, resisting breakdown by environmental forces or *in vivo* metabolic reactions. These properties of HAHs and their widespread presence in the Great Lakes basin elicit concern due to the potential of these compounds to enter all parts of the ecosystem (Langford, 1979).

In laboratory animals, HAHs have been demonstrated to produce a wide range of effects, from anorexia and weight loss to reproductive disruption and death (Borlakoglu and Haegele, 1991). The effects observed vary with the percent chlorination, the position

of the chlorines on the carbon skeleton, and with the species consuming these contaminants. Additionally, the effective dose in 50% of the population (ED_{50}) and lethal dose in 50% of the population (LD_{50}) of the HAHs that have been examined vary widely, both with species and with biological effect.

One of the species most sensitive to the toxic effects of HAHs, the mink (*Mustela vison*), is widely distributed throughout the Great Lakes region, across areas which range from negligible to high contamination with these and similar organic compounds. Based on reports from trappers in the Great Lakes basin, wild mink populations have been declining in contaminated areas over the last 20 to 30 yrs. It has been suggested that contamination of the ecosystem or of the food sources of the mink are in part responsible for this decline (Wren, 1991). A major part of a mink's diet in the wild may be fish, both those that it scavenges from shorelines and those that it catches in streams and lakes. It has been shown that the concentrations of PCBs and other organochlorine chemicals in mink and otters are related to the concentrations of those same compounds in the fish from the territories occupied by these mammals (Foley *et al.*, 1988). The susceptibility of mink to PCB toxicity was first demonstrated in the 1960s, when ranch mink fed diets containing Great Lakes fish experienced reproductive problems, and adult mortality rose (Hartsough, 1965). Investigation of the components of the diet fed to these animals demonstrated that the effects could be linked to the inclusion of Great Lakes fish in the diet (Aulerich *et al.*, 1971). Further research showed that organochlorine contaminants, such as PCBs, were responsible for most, if not all, of the effects observed (Aulerich *et al.*, 1973). The ability of PCBs to affect mink health and reproductive potential has been

demonstrated repeatedly since then, both through the inclusion of PCB-contaminated fish or rabbits in the diets of mink and through the direct administration of PCBs to mink (Aulerich and Ringer, 1977; Bleavins *et al.*, 1980; Shull *et al.*, 1982; Hornshaw *et al.*, 1983; Aulerich *et al.*, 1985, 1986, 1987; Wren *et al.*, 1987a, 1987b).

The overall goal of this study was to determine whether environmental contaminants, such as PCBs, have been involved in the decline of mink populations in the wild. This overall goal was further divided into three primary objectives. The first of these was to determine PCB concentrations in mink exposed to PCBs for up to eighteen mos, either through the diet in adult animals, or *in utero*, via lactation, and through the diet in offspring of exposed animals. This first objective was achieved through the measurement of hepatic and plasma PCB concentrations at the end of the feeding study.

The second objective was to evaluate hepatic cytochrome P450 activities in these same mink. Hepatic cytochrome P450 induction in other animals, such as rats and mice, has become a well-characterized benchmark for PCB exposure. This induction has also been used as an indication of biological activity of PCBs and PCB mixtures, both experimentally and in field conditions (Gillette *et al.*, 1987; Helferich *et al.*, 1987; Nebert, 1991).

The third objective of this study was to determine whether consumption of PCB-contaminated fish by mink would produce some measurable biological change which could be correlated to consumption of increasing concentrations of dietary PCBs.

Hepatic cytochrome P450IA1, which mediates the O-deethylation of ethoxyresorufin (EROD), was selected as the candidate biomarker. As mink are territorial animals, alterations in the activity of cytochrome P450IA1 in wild mink might be used to indicate the extent of PCB contamination of their habitat. Thus, these animals could be used as a sentinel species for the presence of environmental contaminants and to assess the efficacy of remediation efforts.

The fish used in this study were shown to contain contaminants other than PCBs (Restum *et al.*, 1998). Many of the environmental contaminants observed in the diet have not been studied in mink, and therefore their effect is not known. However, some of the non-PCB contaminants found in highest concentration in the fish were p,p'- and o,p'-DDD, p,p'-DDE, and dieldrin (Restum *et al.*, 1998). Previous studies have demonstrated that mink were not affected by up to 100 mg/kg p,p'-DDT and 50 mg/kg p,p'-DDD, that the lethal concentration of dieldrin was well above that contained in the carp used here, and that the Lowest Observable Adverse Effect Concentration (LOAEC) of heptachlor in the diet, in terms of reduced kit growth, was also higher than that in the Saginaw Bay carp used in this study (Aulerich and Ringer, 1970; Crum *et al.*, 1993). The addition of Lake Michigan coho salmon to mink diets produced fatty liver and hepatic degeneration, anorexia, and bloody stools (Aulerich and Ringer, 1977). These signs were similar to those observed in mink treated with PCB congeners (Aulerich *et al.*, 1987; Gillette *et al.*, 1987). Addition of DDT, isomers of DDT, or dieldrin did not produce these same signs, suggesting that the PCBs in the fish were responsible for the adverse health effects (Aulerich and Ringer, 1977). The potential for non-dioxin-like contaminants (DDT,

DDE, dieldrin, nonachlor, heptachlor, and similar compounds) to cause the adverse health effects observed in mink fed Great Lakes fish has also been discussed in Giesy *et al.* (1994a, 1994b). That discussion, taken together with the data outlined above, make it likely that the effects observed in the current study are due to the effects of PCBs and other dioxin-like contaminants in the Saginaw Bay carp.

Fish Collection and Diet Preparation

Carp (*Cyprinus carpio*) were collected from the mouth of the Saginaw River, mixed, and incorporated into diets as described previously (Restum *et al.*, 1997). The fish portion of the control diet contained only ocean fish. Three experimental diets containing 0.25, 0.5, or 1.0 ppm total PCBs were prepared by the substitution of increasing amounts of Saginaw Bay carp for ocean fish in the diet. The composition of the control and experimental diets is shown in Table 1, adapted from Restum *et al.* (1998).

Dietary Assignments and Animal Husbandry

A total of 96 standard dark mink were randomly assigned in December 1991 to one of four dietary groups (0.0, 0.25, 0.5, or 1.0 ppm PCBs), with 16 females and 8 males in each group. These parental (P_1) animals were mated in March 1992 to produce the 1992 F_1 (first filial) kits. When the 1992 F_1 mink were weaned at approximately six weeks of age, half of the P_1 and F_1 animals on the PCB-containing diets were transferred to the control (0.0 ppm PCB) diet for the duration of the study. P_1 and F_1 animals were then mated within their age and treatment groups in March 1993 to produce the second-year F1 and the F2 generation of kits, respectively. At the end of the breeding season (April 1993), the adult male mink were killed as described below. The adult females and the 1993 kits were killed when the latter reached six weeks of age. The generations,

Table 1. Composition of the diets fed to the mink in this feeding study. Adapted from Restum *et al.*, 1998.

Ingredient	% in each diet			
ppm PCBs :	0.0 ppm	0.25 ppm	0.5 ppm	1.0 ppm
Water	25	25	25	25
Cereal[1]	20	20	20	20
Poultry[2]	20	20	20	20
Eggs[3]	5	5	5	5
Corn Oil[4]	1	0.75	0.5	0
Saginaw Bay Carp :				
1987 Carp (12/91-3/92)	0	2.98	5.95	11.9
1989 Carp (3/92-3/93)	0	4.39	8.75	17.5
1991 Carp (3/93-7/93)	0	5.33	10.63	21.3
Ocean Fish[5] :				
12/91-3/92	30	27.02	24.05	18.1
3/92-3/93	30	25.61	21.25	12.5
3/93-7/93	30	24.67	19.37	8.7

[1]K-40 Mink cereal, XK Mink Foods Inc., Plymouth, WI.
[2]Tyson Foods, Fort Smith, AR.
[3]Supplemented with biotin, 25 mg ; Unites States Biochemical Corp., Cleveland, OH.
[4]Arcola corn oil ; PVO Foods, Inc., St. Louis, MO.
[5]Ocean fish, added to each diet to provide a total of 30% fish in the diet ; Boston Feed Supply, Natick, MA.

sources, and duration of exposure, and number of mink in each generation are shown in Table 2. The timetable of the study is also presented schematically in Figure 1.

During the feeding trial, mink were housed in wire cages with attached wooden nest-boxes. Wood shavings and excelsior were provided for nesting materials. The cages were in open-sided sheds, and mink were thus exposed to ambient temperature, humidity, and light conditions for the duration of the feeding study. The animals were provided fresh food and water ad libitum throughout the study.

Necropsy and Tissue Collection

The adult male mink were killed (CO2) in April, 1993. Females and kits were killed in June and July, 1993, when the 1993 kits were weaned at six weeks of age. In all cases, animals were first anesthetized by intramuscular injection of 0.3 ml (adults) or 0.1 ml (kits) Ketaset (ketamine-HCl, 100 mg/ml; Fort Dodge Laboratories, Fort Dodge, IA). Blood was collected by cardiac puncture for plasma PCB and serum triiodothyronine and thyroxine analysis. The mink were then euthanized with CO2 gas. Livers were removed within five minutes of death, rinsed in ice cold 150 mM Kcl, dried, and weighed. A large portion of each liver was immediately frozen in liquid nitrogen for analysis of hepatic cytochrome P450 enzyme activity, while smaller portions of the liver were frozen for determination of hepatic PCB concentration or fixed in 10% neutral-buffered formalin for histopathological examination (Restum *et al.*, 1997). Liver samples frozen in liquid nitrogen were then stored in liquid nitrogen until cytochrome P450 measurements were conducted.

Figure 1. Timetable of treatments and manipulations of the mink fed Saginaw Bay carp in the feeding trial described here. Adult mink (64 female, 32 male) were placed on diets containing ocean fish (0.0 ppm PCBs) or Saginaw Bay carp to provide 0.25, 0.5, or 1.0 ppm total PCBs in December 1991. These parental (P_1) mink were bred, after three months consumption of PCBs, within their dietary groups. Half of the adult mink and half of the F_1 kits consuming diets containing Saginaw Bay carp were transferred to the control (ocean fish, 0.0 ppm PCBs) diet at weaning, after 6 months exposure for the adults and 6 weeks exposure for the kits. The P_1 and the F_1 mink were bred the following spring, after 15 months and 10 months consumption of fish-containing diets respectively, within their diet and age groups, to produce the second-year F_1 and the F_2 kits. Male P_1 and F_1 mink were killed at the end of the breeding season in April 1993, after 16 months or 11 months consumption of diets containing Saginaw Bay carp respectively. Female P_1, first-year F_1, and second-year F_1 and F_2 kits were killed in May and June 1993, after 18 months, 12 months, and 6 weeks consumption of fish-containing diets, respectively.

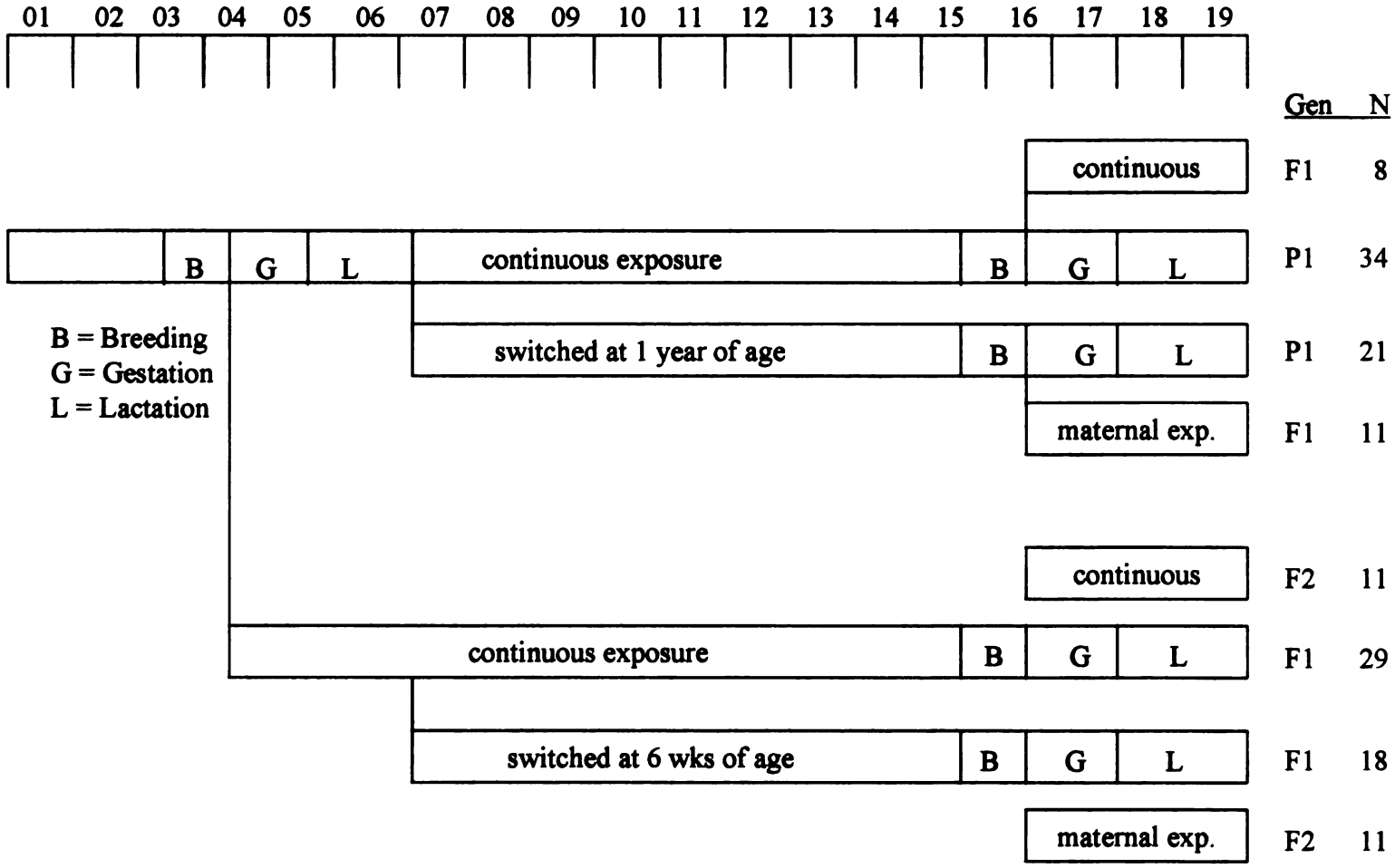
01 02 03 04 05 06 07 08 09 10 11 12 13 14 15 16 17 18 19
Gen N
continuous F1 8
B G L continuous exposure B G L P1 34
switched at 1 year of age B G L P1 21
maternal exp. F1 11
continuous F2 11
continuous exposure B G L F1 29
switched at 6 wks of age B G L F1 18
maternal exp. F2 11
B = Breeding
G = Gestation
L = Lactation

Table 2. Generations, source, and duration of exposure, and number of mink in each dietary group in the feeding study.

Generation	Diet	Exposure	Duration	Female	Male
P_1	Continuous	Diet	18 mo.	34	19
	Switched	Diet	6 mo.	21	12
		Body Burden	12 mo.		
F_1-1	Continuous	P_1 Dam	1.5 mo.	29	12
		Diet	12 mo.		
	Switched	P_1 Dam	1.5 mo.	18	8
		Body Burden	12 mo.		
F_1-2	Continuous	P_1 Dam	1.5 mo.	11	3
	Switched	Switched P_1 Dam	1.5 mo.	11	6
F_2	Continuous	F_1 Dam	1.5 mo.	8	8
	Switched	Switched F_1 Dam	1.5 mo.	11	6

Measurement of Hepatic Cytochrome P450 Activity

Microsomes were prepared by differential centrifugation from portions of each liver as previously described (Helferich *et al.*, 1986). Frozen liver tissue (0.75-1.5 g) was allowed to thaw on ice in 4 ml 19.8 mM Tris/1.5% KCl homogenization buffer, pH 7.4. The liver was minced and then homogenized with a Polytron® homogenizer (Brinkmann Instruments, Westbury, NY) on speed setting 5 for no more than 20 secs. Homogenates were centrifuged for 20 mins at 10,000x g in a Sorvall RC-5B centrifuge (Sorvall Instruments Inc., Wilmington, DE). The supernatant was filtered through two layers of cheesecloth and centrifuged at 105,000x g for 1 hr in a Beckman L7-65 ultracentrifuge (Beckman Instruments Inc., Indianapolis, IN). The resulting pellet was resuspended in 4 ml 0.39 M sucrose/0.77 mM pyrophosphate buffer, pH 7.6, and homogenized with a Polytron® homogenizer on speed setting 3 for 10 secs. The suspension was then centrifuged at 105,000x g for one hr in the ultracentrifuge. The second pellet was resuspended in 1.5 ml 150 mM KCl and homogenized with a Polytron® homogenizer on speed setting 3 for 5 secs. All steps in the preparation were carried out on ice.

Protein Concentration Measurement. Microsomal protein concentrations were determined by the Biuret method (Gornall *et al.*, 1949). Microsomal suspension (50 μl) was diluted in 950 μl distilled water and 1 ml 6% sodium hydroxide. Biuret color reagent (100 μl 1.19 M sodium bicarbonate/69 mM copper sulfate) was added and the components were mixed. The reaction mixture was allowed to incubate at room temperature for 10 mins. The tubes were centrifuged in a Beckman clinical centrifuge (Beckman Instruments Inc., Indianapolis, IN) for 5 mins at room temperature and 2400x g

to sediment any precipitate. The absorbance of the supernatant was then measured at 540 nanometers against a blank of distilled water with a Varian Cary 3E UV/visible spectrophotometer (Varian Inc., San Diego, CA). Absorbance values were compared to a standard curve of bovine serum albumin in 150 mM KCl, prepared along with each protein concentration determination, and microsomal protein concentrations were calculated by linear regression.

Substrate Metabolism Assays. Three cytochrome P450-specific substrates were used. Each was incubated with the microsomal fraction and an NADPH-generating system. Ethoxycoumarin O-deethylase (ECOD; cytochrome P450IA) activity was determined by the formation of coumarin, while ethoxyresorufin O-deethylase (EROD; cytochrome P450IA1), and benzyloxyresorufin O-dealkylase (BROD; cytochrome P450IIB1) activities were measured by the formation of resorufin. Both coumarin and resorufin formation were quantified spectrofluorometrically. Final concentrations of ingredients in the blank and sample incubation mixtures were: HEPES buffer, pH 7.6, 100 mM; $MgCl_2$, 4 mM; glucose-6-phosphate, pH 7.0, 14 mM (Shull *et al.*, 1982). Glucose-6-phosphate dehydrogenase (0.5 IU) and NADP (0.25 mg) were added to the sample incubations to provide an electron-generating system, but were omitted from the blank incubations. Microsomal protein (0.1 mg microsomal protein) diluted in 150 mM KCl to 1 mg protein/ml was added to the incubation mixture on ice. The mixtures were pre-incubated for 3 mins at 37 C in a shaking water bath. The reaction was initiated by the addition of the substrate (ethoxycoumarin, 0.4 μmole in methanol; ethoxyresorufin and benzyloxyresorufin, 1.25 nmole in DMSO), followed by mixing. The reaction mixtures

were then allowed to incubate for 10 mins at 37 C in a shaking water bath. The reactions were terminated by the addition of 250 μl acetone and 1.25 ml 500 mM Tris, pH 9.8, followed by mixing and replacing the tubes on ice. The tubes were centrifuged in a Beckman clinical centrifuge for 5 mins at room temperature and 1000x g. Abundance of coumarin (for ECOD assay) was determined by measuring the fluorescence of the supernatant at an excitation wavelength of 385 nm and an emission wavelength of 435 nm. Abundance of resorufin (for EROD and BROD assay) was determined by measuring the fluorescence of the supernatant at an excitation wavelength of 535 nanometers and an emission wavelength of 585 nanometers. Coumarin and resorufin standard curves were prepared each time the assays were performed. Fluorescence values for blank and sample incubations were compared to the standard curve, and nmoles of coumarin and pmoles of resorufin produced per min per mg protein were calculated by linear regression.

Statistical Analysis

Statistical analysis was carried out using SPSS 6.13 for Windows (SPSS Inc., Chicago, IL). Mean, standard deviation, and standard error were calculated for all dietary and age groups for induction of cytochromes P450IA, P450IA1, and P450IIB1 (ECOD, EROD, and BROD activity, respectively). Means for each dietary and age group were compared to each other for statistical significance by one-way randomized ANOVA, and groups significantly different from each other at $p < 0.05$ were identified using the Least Significant Differences test following ANOVA analysis.

RESULTS

PCB Concentration

Concentrations of PCBs were measured in serum and hepatic tissue from the 217 mink in this feeding study. In mink continuously consuming Saginaw Bay carp, both liver and serum PCB concentrations were above those measured in control (not exposed to PCBs) mink. In mink transferred to the control diet, and in those kits whose dams or grand-dams were transferred to the control diet after 6 weeks or 6 months PCB consumption, PCB concentrations were elevated over PCB concentrations in control animals, but were lower than concentrations in the livers or serum of mink continuously consuming diets containing Great Lakes fish (Table 3).

P_1 (parental) Mink. PCB concentrations in livers of male P_1 mink fed Saginaw Bay carp during the 16 month exposure period increased in proportion to the PCBs contained in the diet. In the male mink switched to the clean diet after 6 months consumption of Saginaw Bay carp, hepatic PCB concentrations were similar to those in the animals fed the control diet throughout the entire study. Female P_1 mink also exhibited an increase in hepatic PCB concentration with increasing dietary PCB concentrations. Female mink switched to the control diet, however, exhibited lesser hepatic PCB concentrations, resembling those observed in control mink.

F_1-1 (first year, first filial) Mink. Concentrations of PCBs in livers of male F_1-1 mink consuming PCB-containing diets for 12 months increased in a dose-dependent manner.

Table 3. Liver and plasma total PCB concentrations, in parts per billion (ppb), in animals exposed to PCBs in utero, via lactation, and/or through consumption of diets containing Saginaw Bay carp for up to 18 months. Samples were taken from the indicated number of animals and pooled prior to analysis (Restum *et al.*, 1998).

Male Mink :

Diet, ppm PCBs	**Plasma PCB Conc.**		**Liver PCB Conc.**	
P_1	**N**	**PCB Conc.**	**N**	**PCB Conc.**
0.0-0.0	4	3.48	4	68.41
0.25-0.25	4	34.35	4	622.0
0.25-0.0	4	15.08	4	132.8
0.5-0.5	3	ND[a]	3	999.0
0.5-0.0	4	11.67	4	220.4
1.0-1.0	3	69.88	3	1600
1.0-0.0	4	21.87	4	304.5
F_1				
0.0-0.0	4	9.4	4	24.02
0.25-0.25	4	41.72	4	636.7
0.25-0.0	4	3.74	4	25.78
0.5-0.5	4	54.17	4	1458
0.5-0.0	4	5.67	4	32.00
1.0-1.0	0	---	0	---
1.0-0.0	0	---	0	---
F_2				
0.0-0.0	4	5.35	4	20.49
0.25-0.25	4	28.98	4	639.9
0.25-0.0	4	9.13	4	29.5
0.5-0.5	1	45.12	1	190.1
0.5-0.0	4	5.54	4	ND
1.0-1.0	3	65.11	3	170.6
1.0-0.0	4	11.97	4	43.96

[a] ND = No Data ; Although there were animals in this group, total PCB concentrations were not determined.

Female Mink :

Diet, ppm PCBs	Plasma PCB Conc.		Liver PCB Conc.	
P_1	N	PCB Conc.	N	PCB Conc.
0.0-0.0	8	6.10	8	71.92
0.25-0.25	8	64.19	8	979.6
0.25-0.0	8	9.26	8	104.4
0.5-0.5	7	101.8	7	891.0
0.5-0.0	7	ND[a]	7	225.7
1.0-1.0	8	154.6	8	1572
1.0-0.0	7	26.71	7	301.5
F_1				
0.0-0.0	7	1.95	7	116.6
0.25-0.25	5	44.12	5	633.5
0.25-0.0	7	5.84	7	30.31
0.5-0.5	6	75.99	6	960.6
0.5-0.0	6	7.58	6	37.19
1.0-1.0	5	127.4	5	1474
1.0-0.0	4	10.49	4	46.86
F_2				
0.0-0.0	8	3.91	8	15.02
0.25-0.25	8	16.70	8	92.41
0.25-0.0	8	11.49	8	28.69
0.5-0.5	2	14.00	2	463.6
0.5-0.0	ND	ND	4	39.64
1.0-1.0	1	66.67	1	180.7
1.0-0.0	6	ND	6	61.59

[a] ND = No Data ; Although there were animals in this group, total PCB concentrations were not determined.

PCB concentrations in livers of mink switched to the control diet at weaning were similar to those of control mink. PCB concentrations in livers of female F_1-1 mink continuously consuming PCB-containing diets exhibited a dose-dependent increase with increasing PCB consumption. In female mink switched to the control diets at weaning, liver PCB concentrations approximated those measured in control animals.

Second-year Mink Kits. PCB concentrations in livers of continuously exposed male and female kits were greater than in those of controls. This increase followed a general dose-dependent relationship with PCB concentration in the diet. PCB concentrations in livers of kits whose dams were switched after 6 months exposure resemble those of control kits.

Hepatic Cytochrome P450 Activity

The activities of hepatic ethoxyresorufin O-deethylase, ethoxycoumarin O-deethylase, and benzyloxyresorufin O-dealkylase enzymes were measured in mink after 6 weeks to 18 months consumption of diets containing Saginaw Bay carp. Measurements of cytochrome P450 activity are presented in Tables 4 and 5. Ethoxyresorufin O-deethylase (EROD) activity demonstrated the greatest responsiveness to PCB consumption in this study, and therefore is the only enzymatic activity discussed here.

P_1 Mink. EROD activity in male P_1 mink continuously consuming PCBs exhibited a general dose-dependent increase in EROD activity. In the 0.5—0.5 group, EROD activity was significantly induced relative to control activity. This group also exhibited significantly induced EROD activity relative to the 0.5—0.0 group. EROD activity in

mink fed Saginaw Bay carp for 6 months and transferred to control diets for 10 months was not significantly different from EROD activity measured in microsomes from control mink. Female P_1 mink fed diets containing Saginaw Bay carp exhibited a dose-dependent increase in EROD activity. EROD activity was significantly induced in the 1.0—1..0 mink compared to both control mink and the 1.0—0.0 mink. In the P_1 females switched to the control diet after 6 months exposure, hepatic EROD activity was not significantly different from the activity observed in control mink (data from male animals is presented in Table 4, while that from female mink is presented in Table 5).

F_1-1 Mink. A significant induction of hepatic EROD was observed in male F_1-1 mink which had been exposed to PCBs *in utero* and via lactation, as well as through their own consumption of diets containing Saginaw Bay carp. EROD activity was significantly greater in microsomes from mink in the 0.25—0.25 group, compared to activity in mink in either the 0.0—0.0 or 0.25—0.0 groups. EROD activity in hepatic microsomes from mink switched to the control diet after weaning was not significantly different from that of control animals. Liver EROD activity of female F_1-1 mink also exhibited a dose-dependent increase with increasing dietary PCB exposure. EROD activity was significantly greater in the 0.25—0.25 than that of both the control and the 0.25—0.0 groups. In switched animals, EROD activity was not different from that observed in control animals (data from male animals is presented in Table 4, while that from female mink is presented in Table 5).

Table 4. Activity (mean ± S.E.) of three hepatic cytochrome P450 isozymes in male mink exposed to PCBs in utero, via lactation, and/or through consumption of diets containing Saginaw Bay carp for up to 18 months[1].

Dietary Group	N	EROD, pmole/min/mg	ECOD, pmole/min/mg	BPOD, pmole/min/mg
P_1				
0.0-0.0	8	34.2 ± 9.1[a]	0.68 ± 0.15	35.0 ± 15.9
0.25-0.25	4	51.4 ± 28.7[ab]	0.65 ± 0.15	18.1 ± 5.5
0.25-0.0	4	33.9 ± 10.0[a]	0.66 ± 0.26	5.0 ± 3.0
0.5-0.5	4	99.8 ± 32.2[b]	0.80 ± 0.23	21.3 ± 6.7
0.5-0.0	4	15.6 ± 5.1[a]	0.66 ± 0.30	26.6 ± 21.5
1.0-1.0	3	62.6 ± 20.9[ab]	0.84 ± 0.36	22.4 ± 9.8
1.0-0.0	4	27.2 ± 4.6[a]	0.79 ± 0.33	55.9 ± 35.5
F_1-1				
0.0-0.0	4	15.6 ± 3.6[ac]	0.59 ± 0.21	19.1 ± 13.2
0.25-0.25	4	114.9 ± 37.0[b]	0.80 ± 0.22	13.2 ± 3.4
0.25-0.0	4	17.9 ± 5.8[ac]	0.64 ± 0.15	35.6 ± 25.8
0.5-0.5	4	96.0 ± 47.3[ab]	1.00 ± 0.30	84.6 ± 40.6
0.5-0.0	4	13.2 ± 3.5[c]	0.66 ± 0.10	42.0 ± 14.0
1.0-1.0	0	--	--	--
1.0-0.0	0	--	--	--
F_1-2				
0.0-0.0	3	8.8 ± 5.2[a]	0.56 ± 0.04	18.7 ± 8.2
0.25-0.25	1	50.8[a]	0.66	10.4
0.25-0.0	2	11.1 ± 3.8[a]	0.44 ± 0.03	13.9 ± 5.2
0.5-0.5	1	111.5[a]	0.82	15.3
0.5-0.0	2	14.7 ± 0.1[a]	0.48 ± 0.04	9.9 ± 0.2
1.0-1.0	3	130.1 ± 1.6[a]	1.32 ± 0.09	69.6 ± 0.07
1.0-0.0	2	6.9 ± 1.4[a]	0.53 ± 0.06	5.6 ± 3.4
F_2				
0.0-0.0	1	11.7[a]	0.53	6.8
0.25-0.25	2	27.9 ± 10.1[b]	0.65 ± 0.01	13.0 ± 0.7
0.25-0.0	2	18.7 ± 8.2[ab]	0.54 ± 0.02	9.1 ± 3.4
0.5-0.5	0	--	--	--
0.5-0.0	2	15.7 ± 0.3[ab]	0.55 ± 0.03	10.7 ± 0.1
1.0-1.0	0	--	--	--
1.0-0.0	2	10.1 ± 0.9[ab]	0.62 ± 0.07	17.4 ± 2.9

[1] Numbers with different subscripts are significantly different from each other.

Table 5. Activity (mean ± S.E.) of three hepatic cytochrome P450 isozymes in female mink exposed to PCBs in utero, via lactation, and/or through consumption of diets containing Saginaw Bay carp for up to 18 months[1].

Dietary Group	N	EROD, pmole/min/mg	ECOD, pmole/min/mg	BPOD, pmole/min/mg
P_1				
0.0-0.0	13	7.4 ± 3.9[a]	0.64 ± 0.07	5.5 ± 2.4
0.25-0.25	7	20.6 ± 1.7[ab]	0.84 ± 0.20	40.0 ± 18.4
0.25-0.0	7	7.2 ± 2.2[a]	0.55 ± 0.12	15.9 ± 2.1
0.5-0.5	6	41.9 ± 37.1[ab]	0.65 ± 0.38	2.5 ± 0.01
0.5-0.0	7	7.8 ± 0.2[a]	0.40 ± 0.08	2.0 ± 0.1
1.0-1.0	8	47.4 ± 17.9[b]	1.11 ± 0.21	47.8 ± 18.5
1.0-0.0	7	12.1 ± 5.2[a]	0.54 ± 0.19	10.2 ± 4.4
F_1-1				
0.0-0.0	13	12.1 ± 1.9[a]	0.41 ± 0.03	5.5 ± 1.8
0.25-0.25	5	97.4 ± 77.0[b]	0.75 ± 0.21	33.1 ± 8.6
0.25-0.0	8	5.8 ± 2.2[a]	0.55 ± 0.15	28.3 ± 8.4
0.5-0.5	6	27.8 ± 5.6[a]	1.55 ± 0.33	83.3 ± 44.4
0.5-0.0	6	8.2 ± 2.6[a]	0.55 ± 0.07	9.0 ± 4.9
1.0-1.0	5	53.1 ± 8.7[ab]	1.93 ± 0.42	118.5 ± 38.6
1.0-0.0	4	9.2 ± 0.1[a]	0.56 ± 0.09	20.4 ± 0.6
F_1-2				
0.0-0.0	3	1.8 ± 0.3[a]	0.39 ± 0.02	8.5 ± 3.1
0.25-0.25	4	50.1 ± 3.9[b]	0.63 ± 0.02	16.7 ± 2.8
0.25-0.0	4	5.9 ± 2.1[a]	0.51 ± 0.08	12.4 ± 3.9
0.5-0.5	0	--	--	--
0.5-0.0	4	9.3 ± 2.0[ab]	0.53 ± 0.02	14.3 ± 2.8
1.0-1.0	1	23.2	0.53	11.1
1.0-0.0	3	4.7 ± 0.5[a]	0.41 ± 0.01	13.7 ± 1.00
F_2				
0.0-0.0	5	5.2 ± 0.7[a]	0.45 ± 0.03	11.5 ± 2.6
0.25-0.25	4	61.1 ± 1.6[b]	0.71 ± 0.01	12.5 ± 2.4
0.25-0.0	4	8.1 ± 0.9[a]	0.73 ± 0.04	47.6 ± 14.1
0.5-0.5	2	66.7 ± 5.5[b]	0.77 ± 0.13	23.0 ± 3.7
0.5-0.0	4	6.1 ± 1.4[a]	0.59 ± 0.02	18.7 ± 1.2
1.0-1.0	0	--	--	--
1.0-0.0	3	7.3 ± 0.3[a]	0.43 ± 0.01	17.5 ± 0.6

[1] Numbers with different subscripts are significantly different from each other.

F_1-2 Mink. EROD activity in livers of male F_1-2 mink kits in the 1.0—1.0 group was significantly higher than in either the control or the 1.0—0.0 groups. EROD activity was also greater in the 0.25—0.25 and 0.5—0.5 groups than in the control group, although these differences were not statistically significant with only one kit in the two PCB groups. EROD activity in the switched kits was not different from that of the control kits. Female F_1-2 kits that survived to the end of the study and were continuously exposed to PCBs had greater EROD activity than the control kits. No kits survived through 6 weeks in the 0.5—0.5 group, and only 1 kit survived in the 1.0—1.0 group. EROD activity in the 0.25—0.25 group was significantly higher than EROD activity in either the 0.0—0.0 and the 0.25—0.0 groups. Although EROD activity in liver microsomes from the 1.0—1.0 group was greater than that of control, the number of mink kits in the groups was small and the difference was not statistically significant. In female F_1-2 kits born to dams switched to the control diet, EROD activity was not different from that in control animals (data from male animals is presented in Table 4, while that from female mink is presented in Table 5).

F_2 Mink. Hepatic EROD activity of male F_2 kits in the 0.25—0.25 group was greater than that of the sole surviving kit in the 0.0—0.0 group. However, EROD activity of the 0.25—0.25 group was not significantly different from that observed in kits in the 0.25—0.0 group. EROD activity observed in switched kits was not different from that observed in the control kits. In the F_2 female kits whose dams were consuming PCB-containing diets during gestation and lactation, EROD activity was significantly greater than in both the control kits and those kits whose F_1 dams were switched to the control diet at

weaning. EROD activity in kits whose dams were switched to the control diet was not different from that of the control kits (data from male animals is presented in Table 4, while that from female mink is presented in Table 5).

DISCUSSION

The experiments described here were designed to examine the induction of hepatic cytochrome P450 isozymes in mink fed diets containing PCBs from Great Lakes fish,and the possibility of using hepatic cytochrome P450 induction as an indicator of PCB exposure in the mink. Although PCB concentration was measured in hepatic tissues of the mink in this study as an indicator of consumption and storage of PCBs in body tissues, PCB concentration alone may not reflect the impact of the contaminant on health, reproduction, and survival in the mink. Earlier research has demonstrated that metabolized PCBs (PCBs consumed and stored by other animals) produce greater decreases in feed consumption and body weight gains and have a lesser LD50 than do nonmetabolized PCBs fed at the same concentration (Aulerich *et al.*, 1986). Wild mink will more likely be exposed to weathered and/or metabolized PCBs than to the parent chemicals. PCBs and other dioxin-like compounds have been shown to induce specific hepatic cytochrome P450 enzymes in a dose-dependent manner. In assessing the impact of environmental contaminants in mink or other wildlife species, it is important to have a measure of that impact that can be correlated to other health effects. Thus, measuring the induction of cytochrome P450 enzymes in mink that had been fed Great Lakes fish will allow determination of the actual effects, rather than solely the concentration, or the PCBs in the fish. The greatest induction of hepatic cytochrome P450 in response to PCB consumption by mink was that measured as EROD activity (Tables 4 and 5).

EROD activity in livers of P1 male and female mink increased with consumption of PCBs from diets containing Saginaw Bay carp. EROD activity of male P1 mink was greater primarily in the middle PCB consumption groups, with less induction observed at the 1.0 ppm dose. Female P1 mink also exhibited less induction than expected at greater doses. In both females and males, it may be that the older mink are less responsive to induction of EROD activity by PCBs than are yong mink. An alternative explanation for this response is that the mink fed diets containing greater doses of PCBs may have consumed less diet than mink receiving lesser doses of PCBs, and therefore may have been exposed to less PCBs than assumed. However, the greater serum and liver PCB concentrations in these mink indicate that this explanation is less likely than lesser responsiveness in older mink. Feed consumption wan only measured at two time points during this study and was therefore not reported. However, body weights of th kits and the F1-1 mink receiving greater doses of PCBs were generally lower than control kit and F1-1 mink body weights. Decreased body weight in these PCB-exposed mink may have translated to decreased body fat, which in turn could decrease storage of PCBs and allow a greater concentration of PCBs to reach the liver and induce cytochrome P450 enzymes to a greater extent in the kits than in the older mink.

Although both male and female F1-1 mink exhibited significant induction of EROD activity when fed Saginaw Bay carp, the greatest induction was observed at less than the maximum dose of PCBs provided in the diet. Decreased feed consumption may again be responsible for the lesser induction of EROD activity by PCBs in the diet.

Alternatively, F1-1 mink may be old enough that, as for the P1 mink, EROD activity is less responsive than in the mink kits.

Male and female F1-2 kits continuously exposed to PCBs exhibited statistically significant induction of EROD activity relative to controls. Continuously exposed F1-2 kits were born to dams that had consumed diets containing PCBs for 16 months prior to mating. The PCB concentrations in the liver of these dams ranged up to 1572 ppb (Table 3). A previous study demonstrated that transfer of PCBs from dams to kits via lactation can occur in the mink (Bleavins *et al.*, 1981). As fat deposits in the dams are used both to provide energy for the dam herself and for transfer into the milk, PCBs stored in fat depots would be released into blood during gestation and lactation. PCBs in the blood of the dams would then have been incorporated into milk and consumed by kits. Thus, through this process, substantial doses of PCBs could be transferred to kits of PCB-consuming dams via milk.

Although the number of kits born in the groups of mink consuming PCBs in this study was decreased, the only significant decrease in kit number was observed in the F1-1 1.0-1.0 generation (Restum *et al.*, 1998). However, survival of the kits in some of the F1-1, F1-2, and F2 kits whose dams were consuming PCBs was also decreased (Restum *et al.*, 1998), as was kit body weight at birth, 3 weeks, and 6 weeks of age in some PCB-consuming groups (Restum *et al.*, 1998). Occurrence of these reproductive effects in generally the groups receiving greater doses of PCBs and other contaminants in the fish suggests that cytochrome P450 induction, which also in general increased with increasing

PCB consumption, can be used to indicate an increased likelihood of effects in animals such as the mink.

The results reported in this study comparing EROD activity in adult mink and kits suggest that younger mink are more responsive to induction of hepatic cyrochrome P450 activity by PCBs than are older mink. These results are similar to a previous study, in which adult mink treated with technical preparations of PCBs (Aroclors) demonstrated only a slight induction of cytochrome P450 activity (Shull *et al.*, 1982). In another study, pregnant mink treated with Aroclor 1254 exhibited 2- to 3-fold EROD induction, while EROD activity in the kits born to these dams increased by as much as 30-fold over EROD activity exhibited in kits born to non-treated dams (Brunstrom, 1991). It was postulated in that report that the induction observed in adult mink was the maximum induction possible, and that younger mink are more sensitive to cytochrome P450 induction than older mink (Brunstrom, 1991). There is therefore not a clear dose-rsponse relationship, in either the P1 or the F1-1 mink consuming PCBs, between dietary PCB concentration and EROD induction. If EROD were adopted as a biomarker for use in the wild, measuring EROD only in adult mink could lead to uncertainty about the resulting prediction of PCB contamination in the habitat. However, if both adult and kit mink were trapped for EROD measurement, then greater induction observed in kit mink in response to dietary and environmental PCBs would lend more weight to the interpretation.

One objective of this study was to determine whether EROD activity would return to preexposure levels when exposure to PCBs ceased. When mink were transferred to the

control diet, EROD activity in these switched mink was not significantly different from that of control mink. This demonstrates that when animals are removed from a source of contamination, at least one of the biological effects produced by that contaminant can decline to preexposure levels.

When a mink is no longer consuming PCBs, the majority of these lipophilic compounds in the body will be partitioned into adipose tissue. Once in fat deposits, PCBs are less available to tissue throughout the body. Many of the efects observed in animals treated with PCBs are mediated through the AhR, a cytosolic receptor that binds PCBs and other HAHs and then acts as a transcription factor to alter gene expression (Denison, 1991). If PCBs are not able to reach the target molecule (AhR), effects mediated through the AhR may decline. This suggests that the PCBs in the liver and other tissues of the mink switched to the control diet are not readily available to the target molecule (AhR).

From the results discussed here, it appears that the induction of EROD activity reflects current, rather than long-term, exposure. As stated earlier, this decrease in cytochrome P450IA1 induction is to be expected when non-PCB-contaminated diet is provided to the animals. Furthermore, these results indicate that EROD activity in the mink is a useful, functional measure of ongoing exposure to coplanar mixtures of HAHs and provides more information than residue concentration alone can provide. Additional studies are required to determine whether biological effects other than EROD induction, such as alterations in reproductive hormone receptor homeostasis, teratogenesis, and

wasting syndrome, are also reduced when the source of contamination is removed from the diet.

The results of this study demonstrate that hepatic cytochrome P450IA1 activity, as represented by EROD activity, is induced by long-term (6 weeks to 18 month) consumption of diets containing environmentally weathered PCBs such as those available in Saginaw Bay carp. Additionally, this is the first study to demonstrate in mink that when the source of PCBs is removed from the diet, hepatic cytochrome P450 activity declines to the level observed in control mink. The response of mink hepatic cytochrome P450 to PCBs, as reported here, is consistent with a 4-month feeding study in mice, in which C57Bl/6 mice were fed diets containing coho salmon from 3 of the Great Lakes. One diet contained approximately 1.0 ppm PCBs through the use of Lake Ontario salmon as 33% (dry weight) of the diet. In that study, EROD activity was induced up to 20-fold over that in groups consuming diets containing Pacific Ocean salmon (Cleland *et al.*, 1987). However, the withdrawal of PCBs from the diet of mice and the effects of this withdrawal on hepatic cytochrome P450 activity were not examined.

Another objective of the study reported here was to determine whether cytochrome P450 enzyme activity could be used as a functional biomarker for PCB exposure in the mink. As mink are territorial animals, alterations in a biomarker in wild mink could be used to indicate the extent of PCB contamination in the area in which the mink were captured, and whether animals were consuming PCBs around the time of capture. Thus, mink could be used as a sentinel species for environmental contaminants and the success of remediation efforts within a given area, if an easily measurable

biomarker existed in mink. For mink to be used as a sentinel speices, however, the biomarker to be used should demonstrate a dose-response relationship with HAH exposure. Ideally, the candidate biomarker would also 1) be easily measurable, 2) exhibit a well-characterized response to HAH consumption, and 3)reflect current rather than long-term exposure to contaminants.

Hepatic cytochrome P450 activity, especially that represented by EROD activity, is such a candidate biomarker. As observed in this study, EROD activity showed a dose-dependent increase with increasing consumption of PCBs through the inclusion of Saginaw Bay carp in the diet. These results are environmentally relevant since the levels of PCBs provided in the diet were similar to those which mink could consume in the wild. PCBs in the diet were accumulated by fish, rather than being artificially elevated doses of technical mixtures or pure congeners of PCBs. The results indicate that a cytochrome P450-based biomarker wold be more useful in younger mink than in adults. However, induction observed even in 2-year old mink suggests that EROD activity could be used as an indicator for PCB consumption. Additionally, decreases in EROD induction in animals switched to control diets suggest that as remediation efforts reduce PCB contamination in mink habitats, activity of this enzymatic biomarker will also decline and thus may only reflect current exposure.

Chapter 3

HEPATIC AND UTERINE ESTROGEN AND UTERINE PROGESTERONE RECEPTOR CONCENTRATIONS

INTRODUCTION

The preceding chapters discussed the decrease in mink populations in areas of the Great Lakes basin and other parts of North America, and the potential involvement of contaminants such as polychlorinated biphenyls (PCBs) in those population declines. The final part of the feeding study described here was designed to examine the possible biochemical mechanisms by which PCBs might cause decreases in mink populations. Studies using ranch-raised mink have demonstrated that diets containing PCB-contaminated Great Lakes fish, technical preparations of PCBs, or pure congeners will decrease or completely block reproduction. For example, this effect has been observed with the addition of fish to diets fed female mink (Aulerich *et al.*, 1973; Hornshaw *et al.*, 1983; Heaton *et al.*, 1995), or the inclusion of such compounds as Aroclor 1254 (Bleavins *et al.*, 1980; Aulerich *et al.*, 1985; Kihlstrom *et al.*, 1992) or Clophen A50 (Kihlstrom *et al.*, 1992) or the pure congener 3,4,5,3',4', 5'-hexachlorobiphenyl (345-HCB) (Aulerich *et al.*, 1985) in the diet. These reproductive effects are observed at extremely low doses, from 0.64 ppm Aroclor 1254 and metabolized PCBs (Hornshaw *et al.*, 1983) to 0.1 ppm 345-HCB (Aulerich *et al.*, 1985). As a recent survey of the contamination by PCBs and other HAHs in common fish in the rivers around the Great Lakes showed (Giesy *et al.*, 1994a, 1994b), the levels which produce reproductive impairment in experimental animal models are far below those which could be ingested from fish. PCBs in the environment

thus have the potential to decrease the reproductive ability of wild mink in contaminated areas throughout the Great Lakes region. Although PCBs also decrease reproduction in other animals, the doses required to achieve the same effect are higher than those required to disrupt reproduction in the mink. Female Sprague Dawley rats were administered Aroclor 1254 at 10 mg/kg/day by oral gavage for at least one month, which included the time period prior to mating and through gestation and parturition. As the rats used in this study weighed 170-230g, this dose schedule resulted in the daily administraton of 2.3 mg Aroclor 1254, and the total administration of 51-69 mg Aroclor 1254. This level of Aroclor 1254 resulted in an 11% decrease in mated females completing pregnancies, compared to 100% completion of mated, untreated rats (Brezner *et al.*, 1984). Female mink were administered Aroclor 1254 at 2 ppm in the diet for 9 months, providing a daily consumption of 0.3 mg Aroclor 1254 (based on 150 g feed consumed per day), and a total dose of 81 mg Aroclor 1254 per mink over 9 months. Reproductive performance in these treated mink was an average of 0.3 kits per mated female, compared to 6.0 kits per female on average in the control group of mink (Aulerich and Ringer, 1977).

PCBs have been shown to decrease cytosolic estrogen receptor concentrations *in vitro*, and to reduce tissue concentrations of estrogen receptor in estrogen-sensitive tissues in rats and mice. It has also been demonstrated that estrogen receptor concentration is decreased in anestrus mink given PCB congeners (Patnode and Curtis, 1994). As liganded estrogen receptor is responsible for the regulation of the estrus cycle and other reproductive processes, decreasing the concentration of estrogen receptor in an estrogen-responsive tissue could decrease the responsiveness of that tissue to estradiol. A

reduction in estrogen receptor concentration has been observed in female mink treated with PCBs (Patnode and Curtis, 1994). The possibility therefore exists that consumption of PCBs by mink produces detrimental effects on reproduction through decreasing the estrogen receptor concentration in estrogen-responsive tissues such as liver and uterus.

The objective of this experiment was to measure hepatic and uterine estrogen receptor concentrations in untreated mink and in mink fed diets containing environmentally relevant concentrations of PCBs for up to 18 months. Additionally, uterine progesterone receptor was measured. Progesterone receptor is one of the proteins whose concentration is regulated by the estrogen receptor and therefore may be useful to indicate the effects of compounds such as PCBs.

METHODS AND MATERIALS

Chemicals and Reagents, Fish Collection, and Diet Preparation

[^{3}H]-17ß-estradiol, [^{3}H]-R5020 (synthetic progesterone), and R5020 were obtained from NEN Radiochemicals (Boston, MA). Hydroxylapatite (HAP) was obtained from BioRad Inc. (Mercury, CA). Carp (*Cyprinus carpio*) were collected from the mouth of the Saginaw River, mixed, and incorporated into mink (*Mustela vison*) diets as described in detail previously (Restum *et al.*, 1998). The fish portion of the control diet contained only ocean fish. Three experimental diets containing 0.25, 0.5, or 1.0 ppm PCBs were prepared by the substitution of increasing amounts of Saginaw Bay carp for ocean fish in the diet. The composition of the control and experimental diets is shown in Table 4 of Restum *et al.* (1998).

Dietary Assignments and Animal Husbandry

A total of 96 standard dark mink were randomly assigned in December 1991 to one of four dietary groups (0.0, 0.25, 0.5, or 1.0 ppm PCBs), with 16 females and 8 males in each group. These P_1 animals were mated in March 1992 to produce the 1992 F_1 kits. When the 1992 F_1 animals were weaned at approximately six wks of age, half of the P_1 and F_1 animals on the PCB-containing diets were transferred to the control (0.0 ppm PCB) diet for the duration of the study. Animals were then mated in March 1993 to produce the second yr F_1 and the F_2 generation of kits. At the end of the breeding season (April 1993), the adult male animals were killed and tissues collected as described below.

The adult females and the 1993 kits were killed when the kits reached six wks of age. See Table 3 and Figure 1 in Restum *et al.* (1998).

During the feeding trial, mink were housed in wire cages with attached wooden nest-boxes. Wood shavings and excelsior were provided for nesting material. The cages were in open-sided sheds, and mink were thus exposed to ambient temperature, humidity, and light conditions for the duration of the feeding study. The animals were provided fresh food and water *ad libitum* throughout the study.

Necropsy and Tissue Collection

The adult male mink were killed (CO_2 asphyxiation) in April, 1993. Females and kits were killed in June and July, 1993 when the 1993 kits were weaned at six weeks of age. In all cases, animals were first anesthetized by intramuscular injection of 0.3 ml (adults) or 0.1 ml (kits) Ketaset (ketamine-HCl, 100 mg/ml; Fort Dodge Laboratories, Fort Dodge, IA). Blood was collected by cardiac puncture for plasma PCB and serum triiodothyronine and thyroxine analyses. The mink were then euthanized with CO_2 gas. Livers were removed within five mins of death, rinsed in ice cold 150 mM KCl, dried, and weighed. A large portion of each liver was immediately frozen in liquid nitrogen for analysis of hepatic cytochrome P450 enzyme activity, while smaller portions of the liver were frozen for determination of hepatic PCB concentration or fixed in 10% neutral-buffered formalin for histopathological examination (Restum *et al.*, 1998). The uterus was removed from each female for estrogen and progesterone receptor concentration

measurements. Uteri were frozen in liquid nitrogen prior to weighing and were subsequently stored in liquid nitrogen until used.

Supernatant Preparation for Estrogen and Progesterone Receptor Concentration Analysis

Hepatic Supernatants. Supernatants were prepared from liquid nitrogen-frozen liver tissues according to previously described methods (DeVito *et al.*, 1994). Frozen mink liver (1.0-1.5 g) was allowed to thaw on ice in 4 ml ice cold TEDG-M buffer (10 mM Tris, 1.5 mM ethylene diamine tetraacetic acid (EDTA), 1 mM dithiothreitol, 10% glycerol, 2 mM sodium molybdate, pH 7.4). The tissue was then homogenized with a Polytron homogenizer (Brinkman Inc., Westbury, NY) on a speed setting no higher than 5 for approximately 20 secs. The homogenate was then centrifuged at 10,000x g for 20 min at 4 C in a Sorvall SS-34 rotor (Sorvall Inc., Wilmington, DE). The resulting supernatant was centrifuged at 105,000x g for 60 min at 4 C in a Beckmann Type 65 rotor (Beckmann Inc., Indianapolis, IN). The final supernatant was carefully removed from beneath the lipid layer and frozen in 0.5 ml aliquots by submersion in a dry ice-isopropanol bath. Aliquots were stored at -80 C until use.

Uterine Supernatants. Supernatants were prepared from liquid nitrogen-frozen liver tissues according to previously described methods (DeVito *et al.*, 1994). Frozen mink uterine tissue (150-200 mg) was thawed on ice in 2 ml ice-cold TEDG-M buffer. The tissue was homogenized and centrifuged as for liver tissues. Following ultracentrifugation, supernatants were removed from the resulting pellet and lipid layer,

transferred to a microfuge tube, and stored on ice until analysis of protein concentration (Bradford, 1976). Estrogen receptor concentration (Erdos *et al.*, 1970), and progesterone receptor concentration (Vickers *et al.*, 1989) were determined as described below.

Bradford Protein Concentration Assay

Protein concentration was determined using the procedure described by Bradford (Bradford, 1976). A standard curve of protein concentrations from 0 μg to 5 μg was prepared using bovine serum albumin in TEDG-M at an original concentration of 0.1 mg albumin/ml. Increasing amounts of this solution were added to distilled water for a final volume of 500 μl. Bradford reagent (500 μl; 0.01% Coomassie Brilliant Blue G-250, 4.7% ethanol, 8.5 % phosphoric acid; Bradford, 1976) was added to each tube. After vortexing, absorbance was measured at 595 nm on a Cary Varian 3E UV/visible spectrophotometer (Varian Inc., San Diego, CA). Mink tissue supernatants were diluted 1:10 into distilled water. Ten μl of this dilution were added to 490 μl water. Bradford reagent (500 μl) was added, the tubes were mixed by vortexing, and absorbance was measured as for the standard curve. All standard and sample tubes were prepared and processed in duplicate.

Scatchard Analysis

Prior to measuring estrogen and progesterone receptor concentration in mink tissues, Scatchard analysis was performed with mink liver and uterine supernatants to verify assay conditions and to determine the K_D and saturation point (B_{max}) of the receptors. The mink liver and uterine tissues used in these assays were obtained from

untreated one- and two-yr old female mink from the MSU Experimental Fur Farm. Either 50 μg supernatant protein (mink uterus) or 100 μg supernatant protein (mink liver) in an incubation volume of 1 ml for a final protein concentration of 0.05 or 0.1 mg protein/ml were used for Scatchard analysis.

Binding of [^{3}H]-17ß-estradiol in a specific and saturable manner was indirectly determined by the subtraction of non-specific binding from total binding of this radioligand to the estrogen receptor. Total binding incubations consisted of supernatant and [^{3}H]-17ß-estradiol in a final incubation volume of 1 ml. For the non-specific binding incubations, diethylstilbestrol (Sigma Chemical Co., St. Louis, MO) was added at a 2,000-fold excess relative to estradiol concentration. Incubations to determine total and non-specific binding were prepared in triplicate at each estradiol concentration. Concentrations of [^{3}H]-17ß-estradiol ranged from 0.1 nM to 20 nM.

Following a two hr incubation at 4 C, 0.5 ml hydroxylapatite (HAP; BioRad Inc., Mercury, CA) suspended in TEDG-M was added. Incubations were held at 4 C for 15 min to allow proteins in the supernatant to bind to the HAP, and were mixed at five min intervals during this incubation. Incubations were then centrifuged at 3,000x g in a Beckmann swinging bucket centrifuge (GH-3.8 rotor) for five min at 4 C. The supernatant was removed, and pellets were rinsed three times with 2.5 ml aliquots of ice-cold TEDG-M. After the third rinse, the pellet was transferred to scintillation vials with 4 ml scintillation cocktail and radioactivity was measured using a Beckmann LS-100 liquid scintillation counter (Beckmann Instruments, Indianapolis, IN).

Scatchard analysis of the progesterone receptor was carried out in the same manner, using 0.1 to 25.0 nM [^{3}H]-R5020 (synthetic progesterone), 1 μM dexamethasone to prevent binding of [^{3}H]-R5020 to the glucocorticoid receptor, and 50 μg supernatant protein in the total binding reactions. For the non-specific binding incubations, R5020 synthetic progesterone was added at a 2,000-fold excess relative to progesterone concentration. Following an 18 hr incubation in a shaking water bath at 4 C, the incubations were stopped by the addition of 0.5 ml HAP in TEDG-M. Incubations were held at 4 C for 15 min to allow proteins in the supernatant to bind to the HAP, and solutions in the incubations were mixed at five min intervals during this incubation. Incubations were then centrifuged at 3,000x g for five min at 4 C in a Beckmann swinging bucket centrifuge (GH-3.8 rotor). The supernatant was removed, and pellets were rinsed three times with 2.5 ml aliquots of ice-cold TEDG-M. After the third rinse, the pellets were transferred to scintillation vials with 4 ml scintillation cocktail and radioactivity was measured with a Beckmann LS-100 liquid scintillation counter (Beckmann Instruments, Indianapolis, IN).

Following Scatchard analysis of [^{3}H]-17ß-estradiol binding to the estrogen receptor in mink liver and uterine tissues and [^{3}H]-R5020 binding to progesterone receptor in mink uterine tissue, estradiol and R5020 concentrations were selected which were above the observed point of saturation. These concentrations were then used for the receptor binding assays.

Estrogen Receptor Concentration Analysis

Incubations using mink hepatic supernatant for determination of estrogen receptor concentration contained 100 μg protein and 20 nM [^{3}H]-17ß-estradiol in a final volume of 1 ml. For the measurement of uterine estrogen receptor concentration, the limited amount of tissue available required the use of only 50 μg protein in each uterine incubation. Uterine incubations were carried out at 20 nM [^{3}H]-17ß-estradiol in a final volume of 1 ml. For both the hepatic and uterine incubations, the labeled estradiol used was diluted to a specific activity of 5.55 Ci/mmole with unlabeled estradiol. Diethylstilbestrol in dimethyl sulfoxide (DMSO) was added to non-specific binding incubations at 2,000-fold excess to estradiol, and an equal volume of DMSO alone was added to the total binding incubations. Both total and non-specific incubations were carried out in triplicate for analysis of each sample, in both uterine and hepatic tissue. As described above for Scatchard analysis, following a two hr incubation at 4 C, 0.5 ml HAP in TEDG-M was added to each tube, incubated at 4 C for 15 mins, and mixed every five min. After centrifugation for five min at 4 C and 3,000x g in a Beckmann model GH centrifuge with a 3.8 rotor, HAP pellets were rinsed three times with 2.5 ml aliquots of TEDG-M. Pellets were then transferred to scintillation vials in scintillation cocktail, and counts were measured with a Beckmann LS-100 liquid scintillation counter.

Progesterone Receptor Binding Assays

Progesterone receptor binding assays were only carried out in mink uterine tissues due to the high concentration of glucocorticoid receptor in the hepatic supernatants, and the ability of this receptor to bind synthetic progesterone as well as its usual ligand. Total

binding incubations contained 50 μg uterine supernatant protein, 20 nM [^{3}H]-R5020, and 1 μM dexamethasone, in a final volume of 1 ml in TEDG-M. The non-specific binding incubations contained a 2,000-fold excess of unlabeled R5020. Total and non-specific binding incubations were carried out in triplicate for each tissue. Tubes were incubated in a shaking water bath at 4 C for 18 hr. Hydroxylapatite in TEDG-M was used to bind protein and protein-bound [^{3}H]-R5020 during a 15 min incubation at 4 C, and the preparation was centrifuged at 3,000x g for five min at 4 C in a Beckmann GH model centrifuge with a 3.8 rotor. The resulting pellets were then washed three times with 2.5 ml aliquots of TEDG-M. After washing, the HAP was then transferred to scintillation vials and radioactivity was measured.

Statistical Analysis

Statistical analysis was carried out using SPSS 6.13 For Windows (SPSS Inc., Chicago, IL). Mean, standard deviation, and standard error were calculated for all dietary and age groups for liver and uterine estrogen receptor concentration. Means for each dietary and age group were compared to each other for statistical significance by one way randomized ANOVA, and groups significantly different from each other were identified using the Least Significant Difference test following ANOVA analysis.

RESULTS

Hepatic Estrogen Receptor Concentrations

Hepatic estrogen receptor concentrations were measured in all female mink used in the exposure study. The concentrations of hepatic estrogen receptor are presented in Table 6.

P_1 Mink. Hepatic estrogen receptors showed a dose-dependent decrease in mink continuously exposed to PCBs through their consumption of Great Lakes fish. Hepatic estrogen receptor concentrations were significantly decreased in the 0.5—0.5 and 1.0—1.0 groups when compared to the 0.0—0.0 group as well as to their respective switched groups. The 0.5—0.5 group showed the greatest decrease, to 40% of the hepatic estrogen receptor concentration in the control animals. The switched animals did not show a significant decrease in hepatic estrogen receptor concentration.

F_1 Mink. Hepatic estrogen receptor concentrations in the F_1 mink did not change with continuous consumption of diets containing Great Lakes fish. Estrogen receptor concentrations were not decreased in the livers of mink transferred to control diets after 15 months consumption of the diets containing Great Lakes fish.

F_1-2 Mink. In the F_1-2 mink, the continuously exposed animals showed a dose-dependent decrease in hepatic estrogen receptor concentrations. The kits whose dams

Table 6. Hepatic and uterine estrogen receptor concentration (mean ± S.E.), in picomoles [^{3}H]-17ß-estradiol bound/mg protein in female mink exposed to PCBs in utero, via lactation, and/or through consumption of diets containing Saginaw Bay carp for up to 18 months[1].

Diet	N	Hepatic ER, pmoles/mg	Uterine ER, pmoles/mg
P_1			
0.0-0.0	13	0.3323 ± 0.0843[a]	0.2117 ± 0.0782
0.25-0.25	7	0.2346 ± 0.0610[a]	0.2102 ± 0.0491
0.25-0.0	7	0.2871 ± 0.0663[a]	0.3689 ± 0.1003
0.5-0.5	6	0.1237 ± 0.0448[b]	0.1667 ± 0.0684
0.5-0.0	7	0.2860 ± 0.0481[a]	0.2335 ± 0.0731
1.0-1.0	7	0.1390 ± 0.0569[b]	0.2584 ± 0.0823
1.0-0.0	7	0.2831 ± 0.0186[a]	0.1903 ± 0.0561
F_1-1			
0.0-0.0	13	0.3099 ± 0.0730[a]	0.3040 ± 0.0956
0.25-0.25	5	0.2897 ± 0.0438[a]	0.2461 ± 0.0645
0.25-0.0	8	0.3023 ± 0.0586[a]	0.2505 ± 0.0538
0.5-0.5	6	0.2653 ± 0.0601[a]	0.3289 ± 0.0981
0.5-0.0	6	0.2834 ± 0.0503[a]	0.2617 ± 0.0495
1.0-1.0	5	0.2749 ± 0.0492[a]	0.2975 ± 0.0864
1.0-0.0	4	0.2795 ± 0.0485[a]	0.2639 ± 0.0320
F_1-2			
0.0-0.0	3	0.2441 ± 0.0395[a]	0.4753 ± 0.1520
0.25-0.25	4	0.1927 ± 0.0640[a]	0.3306 ± 0.0848
0.25-0.0	4	0.2072 ± 0.0728[a]	0.3053 ± 0.0969
0.5-0.5	1	0.1031	0.4019
0.5-0.0	4	0.1568 ± 0.0293[a]	0.3292 ± 0.1052
1.0-1.0	1	0.0748	0.2861
1.0-0.0	3	0.0393 ± 0.0181[a]	0.3712 ± 0.0818
F_2			
0.0-0.0	5	0.4121 ± 0.0762[a]	0.3893 ± 0.1366
0.25-0.25	4	0.3124 ± 0.0583[a]	0.3900 ± 0.0943
0.25-0.0	4	0.3447 ± 0.0530[a]	0.3438 ± 0.0805
0.5-0.5	2	0.1782 ± 0.0542[a]	0.2651 ± 0.0764
0.5-0.0	4	0.2943 ± 0.0626[a]	0.2967 ± 0.0827
1.0-1.0	1	0.1316	0.3829
1.0-0.0	3	0.2539 ± 0.0491[b]	0.3514 ± 0.1465

[1] Numbers with different subscripts are significantly different from each other.

were switched to the control (ocean fish) diet after 15 months consumption of the Great Lakes fish diets also demonstrated a decrease in hepatic estrogen receptor concentration. This decrease was statistically significant when 1.0—0.0 kits were compared to the 0.0—0.0 kits. The hepatic estrogen receptor concentration in continuous and switched kits at each PCB concentration (i.e. 0.25—0.25 vs. 0.25—0.0, 0.5—0.5 vs. 0.5—0.0, and 1.0—1.0 vs. 1.0—0.0 comparisons) were not significantly different in this generation.

F_2 Mink. Both the continuously exposed and the switched F_2 kits exhibited decreased hepatic estrogen receptor concentrations compared to control animals. Kit survival was low in the continuously exposed kits, with only two kits in the 0.5—0.5 group, and only one in the 1.0—1.0 group alive at the conclusion of the feeding trial. The 0.5—0.5 kits had a significant reduction in hepatic estrogen receptor concentration compared to the control kits, although it was not significantly different from that exhibited in the 0.5—0.0 kits. Although there was a dose-dependent decrease in hepatic estrogen receptor concentrations in the switched kits at all dose levels, only the decrease observed in the 1.0 1.0 kits was significantly different from that of control (0.0—0.0) kits.

Uterine Estrogen Receptor Concentration

Uterine estrogen receptor concentrations were measured in supernatants prepared from uterine tissue of all female mink in the exposure study. Estrogen receptor concentrations did not change with consumption of diets containing Great Lakes fish in any of the groups examined. Results are presented in Table 6.

Uterine Progesterone Receptor Concentrations

Uterine progesterone receptor concentrations did not change in any of the groups of mink in this study. Progesterone receptor concentrations in each group are presented in Table 7.

Table 7. Uterine progesterone receptor concentration (mean ± S.E.), in picomoles [^{3}H]-R5020 bound/mg protein in female mink exposed to PCBs in utero, via lactation, and/or through consumption of diets containing Saginaw Bay carp for up to 18 months[1].

Diet	N	Hepatic ER, pmoles/mg
P_1		
0.0-0.0	13	0.4015 ± 0.1678
0.25-0.25	7	0.3692 ± 0.1243
0.25-0.0	7	0.4138 ± 0.1397
0.5-0.5	6	0.3743 ± 0.1164
0.5-0.0	7	0.3985 ± 0.0956
1.0-1.0	7	0.3900 ± 0.1030
1.0-0.0	7	0.3268 ± 0.0856
F_1-1		
0.0-0.0	13	0.2814 ± 0.0756
0.25-0.25	5	0.3170 ± 0.0931
0.25-0.0	8	0.2989 ± 0.0543
0.5-0.5	6	0.2912 ± 0.0721
0.5-0.0	6	0.3304 ± 0.0834
1.0-1.0	5	0.3080 ± 0.0560
1.0-0.0	4	0.2687 ± 0.0682
F_1-2		
0.0-0.0	3	0.3291 ± 0.0942
0.25-0.25	4	0.3406 ± 0.1075
0.25-0.0	4	0.2931 ± 0.0897
0.5-0.5	1	0.3130
0.5-0.0	4	0.3523 ± 0.0926
1.0-1.0	1	0.3341
1.0-0.0	3	0.3201 ± 0.0813
F_2		
0.0-0.0	5	0.2875 ± 0.0504
0.25-0.25	4	0.2786 ± 0.0719
0.25-0.0	4	0.3023 ± 0.0853
0.5-0.5	2	0.3187 ± 0.0847
0.5-0.0	4	0.2961 ± 0.0635
1.0-1.0	1	0.2537
1.0-0.0	3	0.2904 ± 0.0631

DISCUSSION

This study examined the effects of dietary exposure of mink to PCBs, through the addition of Saginaw Bay carp to the diet, on estrogen and progesterone receptor concentrations. A number of studies in mink have demonstrated that the addition of Great Lakes fish to the diet of adult mink decreases the occurrence of estrus and the number of kits born in a given breeding season (Aulerich *et al.*, 1973, 1985; Aulerich and Ringer, 1977; Bleavins *et al.*, 1980; Hornshaw *et al.*, 1983; Kihlstrom *et al.*, 1992; Heaton *et al.*, 1995). In this study, consumption of PCB-contaminated Saginaw Bay carp for up to 16 months led to some slight but generally non-significant trends in reproductive endpoints (Restum *et al.*, 1998).

Measurements of the hepatic estrogen receptor concentration in mink fed PCBs suggest that PCBs reduce estrogen receptor concentration in mink hepatic tissue. The F1-1 females did not display a dose-dependent decrease in estrogen receptor concentration, while the other three generations of animals did.

The reason for the resistance of the F1-1 females to the antiestrogenic properties of the PCBs in the diet is unclear. The estrogen receptor concentration in their offspring, the F2 animals, was reduced in liver supernatant. Although these kits were able to consume solid food after 3 weeks of age, their main food source was from their dams' milk. Previous studies have demonstrated that transmission of PCBs through the milk is

very efficient (Bleavins *et al.*, 1981). The reason for the absence of any effect on estrogen receptor concentrations in the F1-1 dams is unclear.

A comparison of the data in Table 6 shows a greater decrease in estrogen receptor concentration compared to control in the F1-2 0.5-0.5 and 1.0-1.0 kits than in the same groups in the F2 kits. This result is to be expected given the relative length of consumption of PCBs by the dams of these two generations. Because the P1 dams of the F1-2 kits had been consuming PCBs for 16 months prior to whelping the kits, they had an opportunity to accumulate higher concentrations of PCBs than the F1-1 dams of the F2 kits. During gestation and lactation, the PCBs stored in the fat depots of the dams would have been released into the bloodstream, and would have been available for excretion via the milk. Plasma PCB concentrations measured in the P1 and F1 dams at the end of the study demonstrated that plasma PCB concentration was higher in the P1 dams in all exposure groups than in the F1 dams (Restum *et al.*, 1998). Higher plasma PCB concentrations would then lead to increased PCB concentrations in the milk, as PCBs have been shown to enter the milk in mink (Bleavins *et al.*, 1981). This increased consumption of PCBs by the second litter of kits born to the P1 dams could then have resulted in a greater effect on estrogen receptor concentration than in the kits born the the F1 dams.

It was proposed that the decrease in estrogen receptor concentration would have produced a decrease in the concentration of other estrogen-responsive proteins such as the progesterone receptor. Although hepatic estrogen receptor concentrations did decrease

with increasing PCB consumption, hepatic progesterone receptor concentrations were too low to mesure in any of the female mink on this study. As uterine estrogen receptor concentrations did not decrese, no change in uterine progesterone receptor concentration was expected. The accumulation of PCBs in the livers of the female mink exposed to PCBs may account for the decrease in estrogen receptor concentration in the liver and not in the uterus. Since the uterus contains little fat, the concentratons of PCBs in that tissue were probably low. Low uterine PCB concentrations could be responsible for the absence of any decreased estrogen recpetor concentration in the uterus in mink fed diets containing PCBs.

No overall antiestrogenic effect from dietary PCBs was observed in these studies. One potential explanation for the lack of an antiestrogenic response in all generations of mink in this feeding study, and in all tissues examined, is the possibility that some of the PCB congeners contained in the Saginaw Bay carp may have had estrogenic effects. One congener which could have been estrogenic in the model examined here is 3,4,3',4'-TCB. This congener has been demonstrated both in vivo and in vitro to have estrogenic effects. Among these effects are the ability to bind to the estrogen receptor, to upregulate gene expression by the estrogen receptor, and to increase uterine weight in the immature mouse (Nesaretnam *et al.*, 1996). This congener was detected in the fish used for this feeding study, although its contribution to the overall PCB contamination level is small (Restum *et al.*, 1998). Further research with 3,4,3',4'-TCB alone in the mink, and examination of its effect on estrogen receptor, progesterone receptor, and reproductive potential, could contribute to more clearly defining the effects of this congener on reproductive processes.

Identification of other congeners that act as estrogens in in vitro and in vivo systems, and characterization of environmental contaminants as a ratio of estrogenic and antiestrogenic congeners, may resolve this issue.

There are several mechanisms by which PCBs could have antiestrogenic effects. Among these are 1) a reduction of estrogen concentration, 2) a reduction in estrogen receptor concentration, and 3) a competition for the estrogen receptor between estrogen and either the PCB congener or its hydroxylated metabolites. The first proposed mechanism, the reduction of estrogen concentration, suggests that the AhR-mediated induction of cytochrome P450 metabolism, especially the induction of cytochrome P450IA1 , is responsible for increased degradation of circulating 17ß-estradiol (Spink *et al.*, 1990, 1991). If circulating estradiol is decreased, then the effects of this hormone on reproductive tissues will be decreased.

However, several in vitro studies have demonstrated that the antiestrogenic effects of PCBs are observed at concentrations of PCBs that do not observably induce cytochrome P450IA1 (Chaloupka *et al.*, 1992; Zacharewski *et al.*, 1992; Krishnan and Safe, 1993). These observations suggest that reduction of circulating estradiol is not the primary mechanism by which PCBs are antiestrogenic.

The second suggested mechanism by which PCBs exert their antiestrogenic effects is the reduction of estrogen receptor concentrations in estrogen-responsive tissues. If estrogen receptor concentrations are decreased, then the ability of estradiol to elicit its

normal response may be reduced. The antiestrogenic functions of PCBs have been shown to be dependent on a functional (capable of DNA-binding) AhR, by the use of mutant Hepa1c1c7 cells with non-functional Ah receptors. A wild-type strain and two mutant strains were used, one of which possesses an AhR incapable of binding ligands, and one of which possesses an AhR able to bind ligand but lacks the proteins necessary for the ligand-bound receptor to form a DNA-binding complex. In the wild-type Hepa1c1c7 cells, addition of 2,3,7,8-TCDD to the culture medium decreased estrogen receptor concentration in a dose-dependent manner. In the two mutant strains, the addition of 2,3,7,8-TCDD had no effect on estrogen receptor concentration, strongly indicating that the binding of the AhR to both its ligand and to DNA is needed for the 2,3,7,8-TCDD-mediated downregulation of the estrogen receptor (Zacharewski *et al.*, 1991).

The involvement of the AhR in the decrease of cellular estrogen receptors has also been demonstrated in studies in which the structure-activity relationship of the AhR ligands for Ah agonist activity was correlated with the ability of these compounds to decrease estrogen receptor concentrations (Harris *et al.*, 1990). Compounds with high affinity to the AhR elicited the greatest reduction in cellular estrogen receptor concentration in MCF-7 cells, as measured both by radioligand ([3H]-17ß-estradiol) binding, and by immunological detection of the receptor itself (Harris *et al.*, 1990). A decrease in radioligand binding to the estrogen receptor might be accounted for by some confomrational change in the estrogen receptor that was induced by the AhR ligand. This change would then render the estrogen receptor unable to bind ligand. While binding of antiestrogen receptor antibodies cold also be decreased by a conformational change,

decreased detection of estrogen receptor by both immunological detection and radioligand binding suggests that actual estrogen receptor is decreased.

The final proposed method of antiestrogenic activity of PCBs is the competition with estrogen for binding to the estrogen receptor by PCBs or hydroxylated PCBs. Cytochrome P450 metabolism of PCBs can produce hydroxylated PCB molecules, some of which have been shown to have a weak affinity for the estrogen receptor (Korach *et al.*, 1988). In a more recent study, the antiestrogenic activity of 13 hydroxylated PCBs and their ability to decrease estrogen binding to the estrogen receptor were examined in MCF-7 cells. Only 2 of the 13 were shown to mediate their antiestrogenic effects through the estrogen receptor, although they had only weak estrogen receptor binding potencies compared to estradiol itself (Kramer *et al.*, 1997). As mink occupy a relatively high trophic level and thus could consume greater amounts of hydroxylated PCBs from their prey, the ability of hydroxylated PCBs to act as antiestrogens deserves further investigation. This would include examining their ability to act as antiestrogens in vivo as well as in vitro, and determining the exact mechanism for their antiestrogenicity.

The mixed results obtained in this study (decrease in hepatic estrogen receptor concentration in only some generations, no decrease observed in uterus in any groups) and the multiple mechanisms by which PCBs may act as antiestrogens are deserving of further study. The ability of hydroxylated PCB metabolites to compete with estradiol for the estrogen receptor in vivo and their ultimate effect on reproductive outcomes require further investigation. Additionally, determination of the relative contributions of

different congeners to the ultimate estrogenic or antiestrogenic effect of an environmental mixture will help to clarify the potential effect of exposure on wildlife and humans.

Chapter 4

SUMMARY AND CONCLUSIONS

The research presented here reports a general dose-response relationship between ongoing PCB consumption and hepatic cytochrome P450 induction in both male and female mink. Once animals are transferred from PCB-containing to non-contaminated diets, this induction disappears and hepatic cytochrome P450 activity is close to that observed in animals never fed diets containing PCBs. This suggests that hepatic cytochrome P450 activity can be used as an indicator of current PCB consumption in the mink. Hepatic cytochrome P450 activity, through development of either non-invasive methods of measuring cytochrome P450 activity in the mink (caffeine or other substrates whose metabolites are excreted in the urine) or of minimally-invasive methods such as liver biopsy of anesthetized animals to obtain hepatic tissue for cytochrome P450 activity measurement, could therefore be used to indicate the biological effects at a high trophic level of any PCBs in an environment in which mink live.

An additional goal for environmental toxicology and ecological health is the prediction of reproductive capability of wildlife. This can be partially fulfilled by clarifying the mechanism by which PCBs cause decreases in estrogen receptor concentration. Further research should also examine any correlation between decreased hepatic and/or uterine estrogen receptor concentration and decreased reproductive capacity.

Another avenue of experimentation should include examining potential non-invasive or minimally invasive markers for antiestrogenic activity of contaminants such as PCBs. In the area of immunotoxicology, beta 2-microglobulin has been postulated for a marker for the immunotoxic effects of PCBs (Langer *et al.*, 1997). This protein can be easily measured in serum. If a protein was identified that could be used as a marker of PCB exposure and their antiestrogenic activity, then predictions for wildlife could once again be made based on concentrations of that marker in serum.

The new focus on endocrine disruptive effects of environmental contaminants, and the decrease in environmental contaminants to non-lethal levels that may still cause subtle effects in both wildlife and humans, points out the importance of research such as that described above. This research would help in extrapolating the probable effects of environmental contaminants in humans from their effects in wildlife.

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