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DEVELOPMENT OF BEHAVIORAL TESTS FOR MINK: ASSESSING THE NEUROTOXICITY OF POLYCHLORINATED BIPHENYLS AND METHYLMERCURY IN MINK EXPOSED IN UTERO AND DURING LACTATION

presented by

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has been accepted towards fulfillment
of the requirements for

Master's degree in Animal Science

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Date 5/6/98

DEVELOPMENT OF BEHAVIORAL TESTS FOR MINK: ASSESSING THE NEUROTOXICITY OF POLYCHLORINATED BIPHENYLS AND METHYLMERCURY IN MINK EXPOSED *IN UTERO* AND DURING LACTATION

By

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A THESIS

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

MASTER OF SCIENCE

Department of Animal Science

1998

ABSTRACT

DEVELOPMENT OF BEHAVIORAL TESTS FOR MINK: ASSESSING THE NEUROTOXICITY OF POLYCHLORINATED BIPHENYLS AND METHYLMERCURY IN MINK EXPOSED *IN UTERO* AND DURING LACTATION

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Studies have implicated polychlorinated biphenyls (PCBs) and methylmercury (MeHg), persistent environmental contaminants, in causing neurobehavioral deficits observed in young animals exposed developmentally. The objective of this study was to develop behavioral tests for neonatal and prepubertal mink to assess developmental neurotoxicity of environmental contaminants. Adult female mink were fed diets that contained 0.5 ppm PCBs or 0.5 ppm MeHg and were mated to untreated males. The kits were assessed for righting ability, tail-pinch response, eye-opening age, forelimb grip strength, open-field activity, gait measurements, learning ability, and stereotypic behavior. No significant differences were found between treated kits and control kits for any test. The data suggested that the neurological development of the kits exposed to PCBs was delayed and the development of those exposed to MeHg was accelerated. Modifications in these tests should improve their sensitivity to detect behavioral deficits in mink.

Dedicated to the memory of my cousin, Pamela Ruth Heil. You are missed.

ACKNOWLEDGEMENTS

Many people supported my graduate career. First, I thank my major professor, Dr. Richard Aulerich, for his guidance, encouragement, and patience. Thanks also go to my committee members, Drs. Steven Bursian, Christine Williams, and James Sikarskie. Your faith and friendship strengthened me.

I would have floundered without assistance from these people in data collection: Debbie Powell, Chris Stallman, Marsha Morgan, Lorin Stewart, Laurie Grome, Becky Hix, James Martin, Sean Heenan, and Mary Zaloga. My co-workers, Jeff Greenlee, Dianne Karsten, Janine Langham, and Phil Summer, supported me by being flexible when my research schedule was not. My supervisor's help mixing the diets that long day in January and his support of my education is greatly appreciated. Thanks, Angelo.

Secretarial support was provided by Bonnie Cleeves and Carol Daniel. Videotape of stereotypic mink behavior was supplied by Lori Brundige. Kathleen Stewart cut and spliced videotape for me. Ruth Brunke lent me her metronome. Thank you all for your help.

I am especially grateful to Bob Ceru, Dave Erickson, and Nathan Schuck, from the Office of Radiation, Chemical, and Biological Safety, for their direction in safe handling of the methylmercuric chloride. Kristi Sneed and Dr. Richard Rech, from the Department of Pharmacology and Toxicology, were very helpful in my maze work. Dr. Richard Balander helped me with the Power Point software for diagrams and slides. Dr. Martin Balaban,

Department of Zoology, and Dr. Adroaldo Zanella gave me much to ponder in our discussions about behavior. Dr. Robert Tempelmann was indispensable during statistical analysis. Many thanks.

I am indebted to the Department of Animal Science, the Mink Farmers Research Foundation, and especially to Mrs. P. J. Schaible for their support of my research.

I thank my parents for their unfailing love and support.

And Dennis, I could not have done this without you to lean on, cry on, laugh with, dream with, love. I thank you and love you.

Lastly, I thank God for the opportunity to serve Him and for blessing my life.

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INTRODUCTION

Polychlorinated biphenyls (PCBs) and methylmercury (MeHg) are common, persistent environmental contaminants. Laboratory research and epidemiological studies have implicated these compounds as causing neurobehavioral deficits observed in animals exposed *in utero* or postnatally via lactation. Human exposure occurs occupationally, accidentally, or through consumption of contaminated food sources such as fish. Fish from the Great Lakes and related tributaries have been shown to contain PCBs, MeHg, and other toxic compounds (Giesy *et al.*, 1994a; Heaton *et al.*, 1995a; Tillitt *et al.*, 1996).

Mink (*Mustela vison*) were selected for this study of behavioral toxicology because they are among the most sensitive mammalian species to PCB toxicity (Aulerich and Ringer, 1977). The toxicity of mercury has also been studied extensively in mink (Aulerich *et al.*, 1974; Wobeser *et al.*, 1976; Wren *et al.*, 1987a, b). Mink are in the highest trophic level and therefore their risk of exposure to persistent, lipophilic environmental contaminants is high.

The overall objective of this study was to develop behavioral tests for neonatal and growing mink in order to assess developmental neurotoxicity of PCBs in mink. MeHg was used as a positive control as an attempt to validate the tests. These behavioral assessments should provide information to further evaluate developmental neurotoxins so that hypotheses can be postulated on how altered behavior diminishes survivability of wildlife, especially

OBJECTIVES

The specific objectives of this study were to determine the effects of *in utero* and lactational exposure of mink to PCBs or MeHg on:

1. reflex behavior by testing righting ability and tail-pinch response;
2. biological maturation by recording age of eye opening and testing forelimb grip strength;
3. exploratory behavior and emotionality by analyzing open-field activity;
4. motor ability by analyzing stride length and width;
5. learning ability by training and testing growing mink in a one-unit T-maze;
6. stereotypical behavior by recording occurrences and types of behavior described as stereotypical.

LITERATURE REVIEW

Behavior

Neurotoxicity is an unwanted change in the functional status of the nervous system, including learning and memory (Miller and Eckerman, 1986). Behavior can be defined as the net sensorimotor and integrative processes occurring in the nervous system. An alteration in behavior might be a relatively sensitive indicator of exposure to a neurotoxic compound (Tilson, 1990). A large number of industrial chemicals affect the nervous system adversely. Behavioral effects most frequently recognized and reported in humans following exposure to such chemicals include activity changes, incoordination or unsteadiness, reflex abnormalities, weakness, memory problems, excitability, and restlessness (Anger, 1989).

Behavioral teratology refers to the postnatal effects on behavior of prenatal exposure to a developmental toxicant (Vorhees, 1986). Exposure to a developmental teratogen may not result merely in neural alterations that are maintained throughout life, but rather may alter any long-term prospect for future development, progressing to behavioral anomalies that manifest themselves as the animal matures (Spear, 1990). The behavior of an animal represents the interface of that animal and its environment, the purpose of the behavior being the adaptation of the animal to an ever-changing environment (Weisenburger, 1995).

A functional observation battery for neurobehavioral toxicity testing can be developed and organized according to domains of neurological function. Neuromuscular testing of motor skills detects or measures activity changes, incoordination, weakness, abnormal movement and posture, forelimb grip strength, and righting reflex. Sensorimotor indices are used to detect sensory deficits, pain, and equilibrium disorders. Increased

irritability or reactivity and other changes in central nervous system excitability are measured with arousal or reactivity tests. Cognitive testing measures learning and memory. Physiological parameters such as body weight, age at eye opening, body condition, and autonomic function provide information on general health (Mattsson *et al.*, 1989; Buelke-Sam and Mactutus, 1990; Moser and MacPhail, 1990; Tilson, 1990; Moser *et al.*, 1995; Weisenburger, 1995). The basis for selecting a particular test or tests is that the test demonstrates sensitivity, reliability, and validity (Miller and Eckerman, 1986).

When compared to ferrets, skunks, and cats, mink displayed the most rapid improvement in object-discrimination problems, which suggest they are excellent subjects for studies of complex learning ability (Doty *et al.*, 1967). Although, to my knowledge, mink have not been tested in a T-maze, Haddad *et al.* (1976) have had success using ferrets in a T-maze.

From an animal welfare perspective, an animal's behavior is an indicator of its emotional well-being. Captive animals often perform stereotypies: unvarying, repetitive behavior patterns that have no obvious goal or function (Mason, 1993a). When its environment is modified, an animal detects the discrepancies between expected and observed events and attempts to cope with the new situation by means of the repertoire of behavioral and physiological responses which are characteristic of its species and its individual experiences. If coping is prevented by external or internal limitations, then the animal experiences stress (Cabib, 1993). As stereotypic behavior develops, it becomes less and less dependent on feedback from environmental factors. Physical costs of this behavior include weight loss due to increased activity, sores from repeated contact with the cage walls, or

impact injury (Mason, 1993a). Stereotypies in mink have been described by Bildsøe *et al.* (1990a, b; 1991), Hansen (1993), and Mason (1993b).

Polychlorinated Biphenyls

Polychlorinated biphenyls are mixtures of aromatic chemicals, manufactured by the chlorination of a biphenyl in the presence of a suitable catalyst. The chemical formula of PCBs is $C_{12}H_{10-n}Cl_n$, where n is a number of chlorine atoms from 1 to 10. There are 209 possible congeners of PCBs but only about 130 congeners are likely to be found in commercial products (World Health Organization, 1993). Commercial mixtures of PCBs produced in the United States were given the trade name Aroclor®. A specific mixture was designated by a four-digit number, starting with 10 or 12 and the last two digits indicating percent chlorine content (Risebrough and Brodine, 1970). These commercial mixtures range in color from light yellow to dark yellow, do not crystallize, instead turning into solid resins at low temperatures, have very low electrical conductivity and high thermal conductivity, and are very resistant to thermal breakdown. Because of these physical properties, PCBs have been used as dielectric and heat-exchange fluids (World Health Organization, 1993) and in printer's ink, natural and synthetic rubber, fabric and paper coatings, brake linings, paints, varnishes, waxes, asphalt, adhesives, and resins (Risebrough and Brodine, 1970).

Although banned from industrial application since the early 1980s, PCBs can enter the environment via volatilization from landfills containing PCB-using electrical equipment, sewage sludge, improper disposal or incineration of industrial or municipal waste, and overheating or explosions of transformers or capacitors (Ahlborg *et al.*, 1992). Once

released, PCBs are redistributed between soil, water, and the atmosphere, leading to an alteration in the composition of PCB mixtures in the environment. Despite reductions in production and use, PCBs have become widely distributed in the environment worldwide.

All PCB congeners are lipophilic, easily entering the food chain and accumulating in fatty tissues. Persistence of PCB congeners increases as the degree of chlorination increases. Chlorine substitution positions on the biphenyl ring appear to play a role in biodegradation rate. The degree of bioaccumulation in adipose tissue is determined by duration and level of exposure, chemical structure of the compound, and the position and pattern of substitution (World Health Organization, 1993).

Tilson *et al.* (1979) showed that some adult mice exposed *in utero* to 3,3',4,4'-tetrachlorobiphenyl (TECB) (32 mg/kg via gavage on gestation days 10 through 16) displayed a neurobehavioral syndrome consisting of intermittent stereotypic circling, head bobbing, and hyperactivity. These "spinners" were impaired in forelimb grip strength, ability to traverse a wire rod, visual placement responding, and acquisition of one-way avoidance, and several did not have both eyes open by 65 days of age. TECB-nonspinners had similar characteristics but to a lesser degree.

The offspring of female rats exposed to Fenclor 42 (up to 50 mg/kg via intraperitoneal injection), a commercial mixture of PCBs, displayed significant differences from the control group in the development of cliff-avoidance reflexive behavior, swimming ability, and open-field activity (Pantaleoni *et al.*, 1988). Schantz *et al.* (1995) observed spatial learning deficits in adult female rats following gestational and lactational exposure (via gavage to the dam on gestation days 10 through 16) to three individual *ortho*-substituted

PCB congeners commonly found in human breast milk (32 ppm 2,4,4'-trichlorobiphenyl [TRCB], 16 ppm 2,3',4,4',5-pentachlorobiphenyl [PECB], or 64 ppm 2,2',4,4',5,5'-hexachlorobiphenyl [HCB]). The male rats did not display these deficits. Lilienthal *et al.* (1990) noted alterations in open-field ambulation, active avoidance learning, and operant conditioning in rats exposed pre- and post-natally to Clophen A30 (30 mg/kg via the diet), a technical PCB mixture with a chlorine content of 42%. In a cross-fostering experiment, Lilienthal and Winneke (1991) suggested that prenatal exposure had greater importance than lactational exposure, although there is a greater amount of PCBs transferred through nursing.

Perinatal dietary exposure of rats to low levels of Aroclor 1254 (26 ppm) caused a delay in the ontogeny of negative geotaxis, auditory startle, and air righting in the offspring (Overmann *et al.*, 1987). Maximal electroshock seizure tests showed that the exposure decreased seizure severity also. *In utero* and postnatal dietary exposure to up to 8 mg/kg/day Aroclor 1254 produced a permanent auditory dysfunction in rats (Herr *et al.*, 1996). Maternal exposure to a higher concentration (269 ppm) of Aroclor 1254 in this study negatively affected reproduction and pup survival. In mice pre- and postnatally exposed to Aroclor 1254 (up to 82 ppm via the diet), Storm *et al.* (1981) found a decrease in open-field activity and increased latency in conditioned avoidance response training. Pheasant chicks exposed *in ovo* to a high level of Aroclor 1254 (50 mg/week via capsules) showed significantly different behavior on a visual cliff test than control chicks or chicks exposed to a lower dose (12.5 mg/week) (Dahlgren and Linder, 1971).

Bowman *et al.* (1978) found that offspring of rhesus monkeys exposed to Aroclor 1248 (2.5 ppm via the diet) displayed hyperlocomotor activity and learning retardation as a

function of PCB levels in body fat. The researchers pointed out that the hyperactivity expressed itself only after many exposures to the activity cage, suggesting that the monkeys initially suppressed the hyperactivity through their fear of a novel apparatus but slowly adapted to the cage and lost their fear. This study did experience problems with reproductive success and infant survival, however, which could have affected results obtained from surviving offspring. Reproductive effects were also seen in the study by Allen *et al.* (1980), in which female rhesus monkeys were exposed to Aroclor 1248 (up to 5 ppm via the diet) for 18 months. The monkeys were bred during and after exposure, and both sets of offspring developed signs of PCB intoxication. No behavioral tests were performed. Again, in the study by Mele *et al.* (1986), overt signs of toxicity were manifested in the high-dose (2.5 ppm versus 0.5 ppm, dietary) offspring during lactation. While the high-dose group had greater interanimal variability in fixed-interval response rate than the control group, the authors did not discuss what effects any overt symptomatology might have had on the behavioral differences noted.

Levin *et al.* (1988) stopped dietary exposure of female rhesus monkeys to Aroclor 1248 (2.5 ppm) or Aroclor 1016 (1.0 ppm) one year or seven months, respectively, before conception. They tested the offspring on a spatial learning and memory task and found deficits in performance accuracy. They felt the deficits were due, not to memory impairment, but to impairments in associational or attentional processes. The treated offspring also displayed deficits in discrimination-reversal learning (Schantz *et al.*, 1991). The authors did not discuss any health effects.

Behavioral problems as well as other health effects have been documented in children

of over 2,000 women who consumed rice oil contaminated by heat-degraded PCBs in Yucheng, Taiwan (Gladen *et al.*, 1988; Rogan *et al.*, 1988; Yu *et al.*, 1991; Chen *et al.*, 1992; Lai *et al.*, 1994, Yu *et al.*, 1994; Guo *et al.*, 1995). Health effects included shorter heights and lighter body weights than the non-exposed cohort, ectodermal abnormalities, and type B hepatic porphyria. When subjected to behavioral testing, the exposed children were found to have delayed cognitive development and higher activity levels than controls. Yu *et al.* (1994) indicated that Yucheng children can, as the control children do, learn from their environment to modify their behavior and suggested that the exposed children learn as well and as fast as the controls.

While no research has been done on the developmental neurotoxicity of PCBs in mink, this species has been used for acute and reproductive toxicity studies of polyhalogenated aromatic hydrocarbons. Dietary exposure to 3,3',4,4',5,5'-HCB (0.05 ppm) caused anorexia, bloody stools, disrupted molting patterns, and nail deformities in mink (Aulerich *et al.*, 1987). Mink treated intraperitoneally with 3,3',4,4'-TECB (50 mg/kg) developed anorexia and diarrhea and their small intestines were shown to have a severe necrotizing enteritis (Gillette *et al.*, 1987a, b). Dietary exposure to Aroclor 1254 (1 ppm) did not affect mink fertility but did reduce kit growth during the lactation period (Wren *et al.*, 1987b). When exposed neonatally to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) via intraperitoneal injection (0.1 μg/kg), mink kits experienced a reduction in body weight and, in acute toxicity cases (1.0 μg/kg), developed distended abdomens and ascites (Aulerich *et al.*, 1988). Adult mink receiving a single dose of TCDD (up to 7.5 μg/kg via gavage) showed a dose-dependent decrease in feed consumption and corresponding weight loss

(Hochstein *et al.*, 1988). Necropsies conducted on the animals revealed discoloration of the liver, spleen, and kidneys and higher relative brain, kidney, heart, adrenal, and thyroid weights than the control group.

Rats exposed peri- and postnatally to TCDD (up to 1.0 μg/kg one time, and up to 0.4 μg/kg weekly, respectively, via subcutaneous injection) showed behavioral effects of reduced ability to remain on a rotating rod and increased successful response rate during reflex testing (Thiel *et al.*, 1994) No effects on learning were found in a discrimination learning task.

A key source of environmental exposure to PCBs is the consumption of contaminated fish. Mink, otters, and porpoises are among the mammalian wildlife species found to have organochlorine residues in their tissues (Duinker and Hillebrand, 1979; Henny *et al.*, 1981; Foley *et al.*, 1988; Keymer *et al.*, 1988). Piscivorous, colonial waterfowl of the Great Lakes region have been studied extensively and the effects of dioxins, dibenzofurans, and PCBs on reproductive performance, anatomical development, and nesting behavior evaluated (Giesy *et al.*, 1994a). Anomalies observed include eggshell thinning, deformities, tumors, immune suppression, edema, hormonal changes, enzyme induction, wasting syndrome, and porphyria.

In the laboratory, rats have been fed diets containing salmon (up to 30%) from Lake Ontario and, along with their offspring, have shown behavioral aberrations (Hertzler, 1990; Daly, 1992, 1993). No overt signs of toxicity were noted in these experiments, suggesting that behavioral changes can be effected by doses below the standardized Lowest Observed Adverse Effect Level (LOAEL) or No Observed Adverse Effect Level (NOAEL). The treated rats in these studies displayed reduced activity in an open-field test (Hertzler, 1990) and hyper-reactivity to aversive situations (Daly, 1992, 1993).

Hornshaw *et al.* (1983) determined that PCBs exposed to biological processes prior to consumption are more toxic to mink than corresponding technical mixtures. Mink fed Great Lakes carp failed to reproduce and reproductive success in mink fed other fish or fish products from the Great Lakes was inferior to the control. Heaton *et al.* (1995a, b) found mink fed polyaromatic hydrocarbon-contaminated carp (up to 40%) from Saginaw Bay, Lake Huron to act nervous when approached. Decreased reproduction and survivability were observed in this study as well.

Exposure to the contaminants in Great Lakes fish has been implicated as the cause of behavioral problems experienced by children born to mothers who consumed Lake Michigan sports fish (Jacobson *et al.*, 1984a, b; 1990; Jacobson and Jacobson, 1993). The Jacobson studies suggested intrauterine exposure was more critical than postnatal ingestion due to its continuous nature and the developing fetus's vulnerability to teratogenic agents. Postnatal exposure was not related to any physical growth or cognitive deficits, only to a small reduction in activity level. The researchers did not feel the effects were attributable to lead, polybrominated biphenyls, or certain pesticides, which are also found in Great Lakes fish. Behavioral deficits included hypoactive reflexes, motoric immaturity, a greater amount of startle, and "worrisome" infants. Gross impairment and mental retardation were not increased in the exposed cohort.

Methylmercury

Mercury is a naturally-occurring element. Inorganic mercury, also known as metallic or elemental mercury, is a shiny, silver-white liquid, commonly used in thermometers. When

combined with other elements, such as chlorine or oxygen, inorganic mercury salts are white powders or crystals, except for mercuric sulfide which is red and turns black after exposure to light. Mercury can chemically bond with carbon to form organomercurial compounds, such as methylmercury and phenylmercury. These compounds are white crystalline solids and can also exist as salts (ATSDR, 1994).

Metallic mercury is used in thermometers, barometers, batteries, electrical switches, dental fillings, and the production of chlorine gas and caustic soda. Inorganic mercury compounds and salts have been used in skin lightening creams and fungicides and as antiseptics and pigments. Methylmercury is not generally produced by human activity. Before their ban in the 1970s, methyl- and ethylmercury compounds were used to protect seed grains from fungal infections (ATSDR, 1994).

Both inorganic and organic mercury compounds are found in the environment. The mercury portion of these compounds does not break down into other chemicals. However, either form can be changed to the other by microorganisms and natural processes. Methylmercury is the usual organic form produced and is of particular concern because of its ability to bioconcentrate in fish (ATSDR, 1994). Methylmercury can account for 90% of the total mercury content in the flesh of edible fish (O'Kusky, 1992).

The effects of both inorganic and organic mercury on the developing rat brain have been studied. Fredriksson *et al.* (1992) exposed neonatal rats to metallic mercury vapor (0.5 mg/m^3 for up to four hr) and tested the pups at two and four months of age for spontaneous motor activity and learning in a radial arm maze and in a circular swim maze. The researchers found dose- and age-related behavior changes, specifically, marked hypoactivity

and retarded acquisition in the radial arm maze. There was no difference when compared to controls in acquisition rate in the swim maze. Rats exposed prenatally during organogenesis to methyl mercuric chloride (up to 2.5 mg/kg via gavage to the dam on gestation days 6 through 15) displayed no overt sign of neurotoxicity but were noted to have subtle changes in age at eye opening and in neuromotor coordination and neurochemical profile (Sobotka *et al.*, 1974). In a similar experiment (using up to 2.0 mg/kg via gavage on gestation days 6 through 9), Müsch *et al.* (1978) found significant differences in acquisition speed in a lever-box when female rat offspring were trained to press a bar for a food reward. They did not find differences in general motor ability or motor coordination. The general brain morphology of neurons and glial cells was examined in rats exposed pre- and postnatally to methyl mercury (3.9 ppm via the diet) and were found not to be significantly different from controls (Lindström *et al.*, 1991). No behavioral tests were performed in that study.

In mice prenatally exposed to methylmercury dicyandiamide (8 mg/kg dam weight on gestation day 7 or 9 via intraperitoneal injection), offspring took a longer time to begin exploration in an open field and showed signs of neuromuscular impairment while swimming (Spyker *et al.*, 1972). However, analyses of brain weight, protein enzyme activity, and choline acetyltransferase and cholinesterase activities revealed no significant difference between treated and control offspring. Su and Okita (1976) found decreases in exploratory behavior and spontaneous motor activity in an open field in mice exposed *in utero* to methylmercury hydroxide (up to 12 mg/kg on gestation day 10 via subcutaneous injection).

Daily oral exposure to methyl mercuric chloride (50 μg/kg/day) from birth to seven

years of age impaired high-frequency hearing and affected spatial and temporal visual function in cynomolgus monkeys (Rice and Gilbert, 1992). Overt toxicity was observed at 13 years of age, with some displaying increased clumsiness.

A major outbreak of methylmercury poisoning occurred in Iraq in 1971-72 when methylmercury-treated seed grain was ground into and used as bread flour. Neurological effects were found in some children whose mothers had been asymptomatic during pregnancy. These effects included delayed achievement of developmental milestones, such as walking and talking, with or without neurological signs, such as motor or speech retardation or seizures (Marsh *et al.*, 1980).

Both inorganic and organic forms of mercury have been fed to mink. Aulerich *et al.* (1974) found 10 ppm of supplemental inorganic mercuric chloride did not produce adverse effects, whereas 5 ppm of supplemental methylmercury proved fatal to adult mink in one month. When mink diets were supplemented with 1 ppm methylmercuric chloride alone or in combination with 1 ppm Aroclor 1254 and the mink were housed outside prior to breeding in late winter, unexpected mortality claimed the majority of the females (Wren *et al.*, 1987a, b). At 0.5 ppm methylmercuric chloride with 0.5 ppm Aroclor 1254, no dams died, however kit survival was lower than that for Aroclor 1254-only or control groups. Clinical signs of methylmercury toxicity in mink are anorexia, loss of weight, incoordination, tremors, and convulsions. Wobeser *et al.* (1976) found that mink fed diets containing up to 15 ppm methyl mercury chloride developed clinical signs of intoxication in direct relation to the concentration of mercury in the feed. Dysphonia, irregular vocalization, was observed in one mink each from the 4.8 and 8.3 ppm treatments. To my knowledge, no developmental

behavioral studies with methylmercury on mink have been performed.

MATERIALS AND METHODS

Experimental Diets

There were three diets fed to the mink in this study. Diet A (Control, Table 1) consisted of typical mink feed ingredients and represented the conventional diet fed at the MSU Experimental Fur Farm. Diet B was similar in composition to Diet A except that carp (*Cyprinus carpio*) from Saginaw Bay, Lake Huron was substituted for a portion of the herring meal to provide a targeted concentration of 0.5 ppm PCBs in the diet. Diet C was the same as Diet A except for the addition of methyl mercuric chloride to provide a targeted concentration of 0.5 ppm MeHg.

Premix Preparation

Under a fumehood, 0.2457 g of methyl mercuric chloride (Alfa Aesar, Ward Hill, MA; ≥95% purity) was weighed into a foil dish. The compound was washed from the dish into a beaker using 946.3 ml of 100% ethanol. The solution was stirred with the weighing spatula until all crystals were dissolved. The solution was poured into a stainless steel tray containing one kg ground mink cereal. The spatula and beaker were rinsed with 473.2 ml of ethanol and the rinsate was added to the tray. The slurry was stirred to ensure complete saturation. The tray remained under the fumehood 72 hours to allow the ethanol to evaporate.

The dried mixture was added to four kg ground mink cereal and tumbled in a sealed container for 15 minutes. The total five kg of premix was part of the total cereal in Diet C.

Table 1: Diet Compositions

	Diet A	Diet B	Diet C
Ingredients			
Water, %	27.5	25.7	27.5
Chicken viscera, %[1]	26.0	24.0	26.0
Herring meal, %[2]	7.0	3.6	7.0
Cereal, %[3]	26.0	24.0	26.0
Raw eggs, %[4]	7.0	6.5	7.0
Beef liver, %[5]	6.0	5.6	6.0
Corn oil, %	0.5	----	0.5
Carp, %[6]	----	10.6	----
d-biotin premix, mg/kg[7]	0.05	0.05	0.05
Methyl mercuric chloride, mg[8]	----	----	245.7

[1]Tyson Foods Inc., Ft. Smith, AR
[2]A-B-C Brand, St. Laurent Gulf Products, Ltd., Garaquet, NB, Canada
[3]XK-40 Mink Food, XK Mink Foods Inc., Plymouth, WI
[4]MSU Poultry Farm, East Lansing, MI
[5]Ada Beef Co., Ada, MI
[6]Containing 4.7 ± 1.76 ppm PCB (Restum *et al.*, 1998); collected from Saginaw Bay, MI
[7]Containing 100 mg d-biotin/lb; Feed Specialties, Des Moines, IA
[8]Alfa Aesar, Ward Hill, MI (Lot #G07F44)

Diet Preparation

The experimental diets were prepared using the equipment at the MSU Experimental Fur Farm. The composition of the diets is shown in Table 1. The water, biotin, corn oil (if applicable), Diet C premix (if applicable), herring meal, and cereal were placed, in that order, into a paddle mixer and mixed. The chicken viscera, eggs, beef liver, and carp (if applicable) were ground through a 0.95 cm (3/8 inch) face plate and added to the mixer while it was mixing. Each diet was mixed for 15 minutes. Four samples were removed from each diet and stored frozen (-20°C) in Whirl-paks® for subsequent analyses. The feed was placed in color-coded labelled buckets lined with plastic bags and sealed with lids. The feed was stored frozen until needed for feeding.

Experimental Design and Animal Care

On January 22, 1996, 36 standard dark, one-year-old female mink (*Mustela vison*) were randomly assigned to the three treatment groups. Care was taken so that littermates were not placed within the same treatment group in an attempt to reduce any genetic predisposition to PCB or mercury toxicity or to stereotypic behavior. The adult mink had been vaccinated as kits against canine distemper, virus enteritis, hemorrhagic pneumonia, and botulism (Distox-Plus; Schering-Plough Animal Health Corp., Omaha, NE).

The adult mink were housed individually in wire "breeding" cages (78 cm L x 46 cm W x 38 cm H) with attached nestboxes (34 cm L X 26.5 cm W X 27 cm H) bedded with pine shavings (Pestell Agri-Products, Ontario, Canada). Prior to the female whelping, the nestbox was bedded with aspen shavings (Northeastern Products Corp.,

Warrensburg, NY) to prevent the kits from being exposed to toxic terpenes in the pine shavings, and with "wood wool" excelsior (American Excelsior Company, Arlington, TX). A temporary false floor of 1.27 cm (1/2 inch) wire mesh was fitted to the permanent cage floor to prevent young kits from falling through the 2.54 x 3.81 cm (1 x 1-1/2 inch) wire mesh. A hardboard partition (30.5 cm X 15 cm) was fitted into the nestbox to prevent the kits from crawling out of the nestbox into the cage.

At six weeks of age, the kits were weaned from their dams. The dam was removed from the experiment, and all animals received the basal ("ranch") diet. At seven weeks of age, the litters were divided so that the kits continuing with behavioral testing were housed separately from their siblings. At eight weeks of age, all kits were housed singly. Those continuing with behavioral testing were moved to "grower" cages (61 cm L x 30 cm W x 38 cm H) with penthouse-style nestboxes (30 cm L x 20 cm W x 29 cm H).

Feed and water were provided *ad libitum* to the female mink and their litters throughout the study until the kits were trained for the T-maze test, at approximately 12 weeks of age, when feed was occasionally restricted to the kits in order to motivate them to learn the task.

The adult mink were individually identified, each having an identification card with the mink number, diet letter, and project name. A color-coded tag identifying the diet was fastened to the door of each cage for ease in identifying the appropriate diet during feeding. The kits were individually identified at seven weeks of age, each having a card with the date of birth, gender, project name, dam number, and diet letter.

All animals were observed daily and any behavioral changes or clinical signs of

toxicosis were recorded. A wall fan was operated during the testing to provide constant background noise.

Exposure Period

The exposure period began January 22, 1996. The adult female mink were weighed weekly until the beginning of breeding season in order to monitor overall health.

One container of each diet was removed as needed from the freezer and allowed to thaw overnight at room temperature. The adult mink received approximately 250 g of feed, placed on a wire grid on top of each cage. When the kits were three weeks old, the feed was mixed with water to a gruel consistency and place on a feed plate on the bottom of the cage in front of the nestbox to encourage the kit to begin consuming "solid" feed. At this time, the partition in the nestbox was removed so the kits could move freely from the nestbox into the cage. As the kits became accustomed to eating the feed, it was mixed to an increasingly thicker consistency and fed. After the kits reached seven weeks of age, the feed was placed on the wire grid on top of the cage in an amount appropriate for the number of animals in the cage. Each morning, the previous day's feed was scraped off the grid or feed plate and discarded before fresh feed was provided. The remaining unused feed in the container was stored in a walk-in cooler (5° C) and used the following day.

Because carp contains the enzyme thiaminase which hydrolyses thiamine resulting in Chastek's paralysis, a thiamine supplement was given to the adult mink on all diets while they were on the feeding trial. Each day 0.288 g of thiamine hydrochloride (Sigma Chemical Co., St. Louis, MO; Lot #72H0102) was dissolved in 50 ml of water and mixed

into 750 g of basal farm diet (containing thiaminase-free herring meal). Each adult mink was fed approximately 21 g of the thiamine-supplemented feed daily before the treatment diet was provided, yielding a dose of eight mg of thiamine per animal per day.

Reproduction

Mating of the females to untreated males began March 4, 1996 and ended March 27, 1996. Females were given the opportunity to mate every fourth day until a confirmed mating (presence of motile sperm in a vaginal aspiration) was obtained. Once a confirmed mating was obtained, the female was given the opportunity for additional matings the day following the initial mating and eight and nine days later, a common commercial mink farm practice.

During mating attempts, males were locked out of their nestboxes and a female was introduced into each male's cage. If no evidence of mating was observed within 15 minutes, the female was placed with a different male. If no evidence of mating with the second male was observed within 15 minutes, the female was returned to her cage and given a slash mark on her breeding record for that day. If mating appeared to be occurring, the pair was left alone until they separated. The female was then taken into the main building where a pipet, containing a small amount of warm saline, was inserted into her vagina. An aspiration was taken and placed on a glass slide and examined under a microscope. If motile sperm were found, the female was considered bred and the male's identification number was written on her breeding record for that day. If no sperm or non-motile sperm were found, the female was given the opportunity to mate with a different

male either that day or the following day. Mating attempts were continued through the breeding season until at least two confirmed matings were obtained for each female.

All nestboxes were checked daily during the whelping season for newborn kits. Live kits were sexed and weighed at birth and weekly thereafter until six weeks of age. Any dead (stillborn) kits were sexed and weighed and removed from the nestbox. The dam's body weight was recorded at whelping and three and six weeks later. Any females that did not whelp by May 22 were removed from the experiment.

Righting Ability Test

In order to assess reflex development, the righting ability of the kits was measured at birth and weekly to three weeks of age. The kit was placed in a supine position and released. The time from release until the second foreleg touched the ground was recorded. The time limit was kept to 30 seconds to prevent the kit from overtaxing itself. If the kit could not right itself within the time limit, a "+" was entered into its record for that day's test. A note, "n", was made if a kit was awake and crying but not attempting to right itself. Any kit that was sleeping and could not be awakened for the test received an "s" in its record for that day.

Tail-Pinch Response Test

A second reflex development test, tail-pinch response, was measured at birth and three weeks of age. The kit was placed on its belly. The tail was pinched once with blunt forceps at a point halfway between the tip and the base. The number of responses (0-3)

observed after the pinch was recorded: the kit tucked its tail, the kit vocalized or increased the intensity of its crying, the body or legs displayed a reflex reaction.

Eye Opening Test

As an indicator of physical development, the kits were checked daily for eye opening. The date was recorded when the first kit in a litter opened at least one eye.

Forelimb Grip Strength Test

The forelimb grip strength, an index of motor and coordination skills, was assessed in one kit of each sex chosen at random per litter at six weeks of age. These kits were used for all subsequent testing. The kit was placed sideways on a 2.54 x 2.54 cm (1 x 1 inch) wooden rod and allowed to hang by its forepaws (Figure 1). The rod was 31 cm above the floor which had cushioning material on which the kit could land if it fell. The time from when the kit was released to when it lost its grip on the rod was recorded. Any kit that retained its grip for more than 60 seconds was removed from the rod and received a "+" for that test's record.

Open-Field Test

At six, seven, and eight weeks of age, the kits selected for the forelimb grip strength test were subjected to an open-field test in order that nonforced behavior could be observed. The test area consisted of a wire ring of 1.27-cm (1/2-inch) mesh 108 cm (42.5 inches) in diameter and 61 cm (24 inches) high placed upon a sheet of glassboard on which

Figure 1: Forelimb Grip Strength Test

was painted a numbered grid, the squares measuring 30.5 cm (12 inches) per side (Figure 2). The wall of the ring was lined with black posterboard and the room lighting was decreased during the test to minimize shadows that might distract the kit. A metronome was set to 40 beats per minute. The kit was released at the center of the ring facing north and was allowed to roam freely for three minutes. At each "click" of the metronome, the kit's position on the grid was recorded. The observer stood outside the east side of the ring and the person recording sat out of the kit's sight. Notes were made if the kit urinated, defecated, vocalized, or scratched or jumped at the wall of the ring as an index for evaluating emotionality. The glassboard was washed with a bleach solution prior to each kit's use.

Gait Measurement Test

At six, seven, and eight weeks of age, the kits selected for the forelimb grip strength test were subjected to a gait measurement test, as a second, nonforced behavior test. The kit's hindfeet were pressed against a sponge soaked in red finger paint thinned with water. The kit was then allowed to walk across a 57 cm X 46 cm sheet of white filter paper. A nestbox was placed at the opposite end of the paper from the point of release to encourage the kit to walk a straight line. The distance between two consecutive stride prints was averaged to determine stride length (Figure 3). Stride width was measured between the middle of a print from one foot and the line of the stride from the other foot. The kit's weight was recorded as well. If a kit ran during the test, the results for that test were nullified.

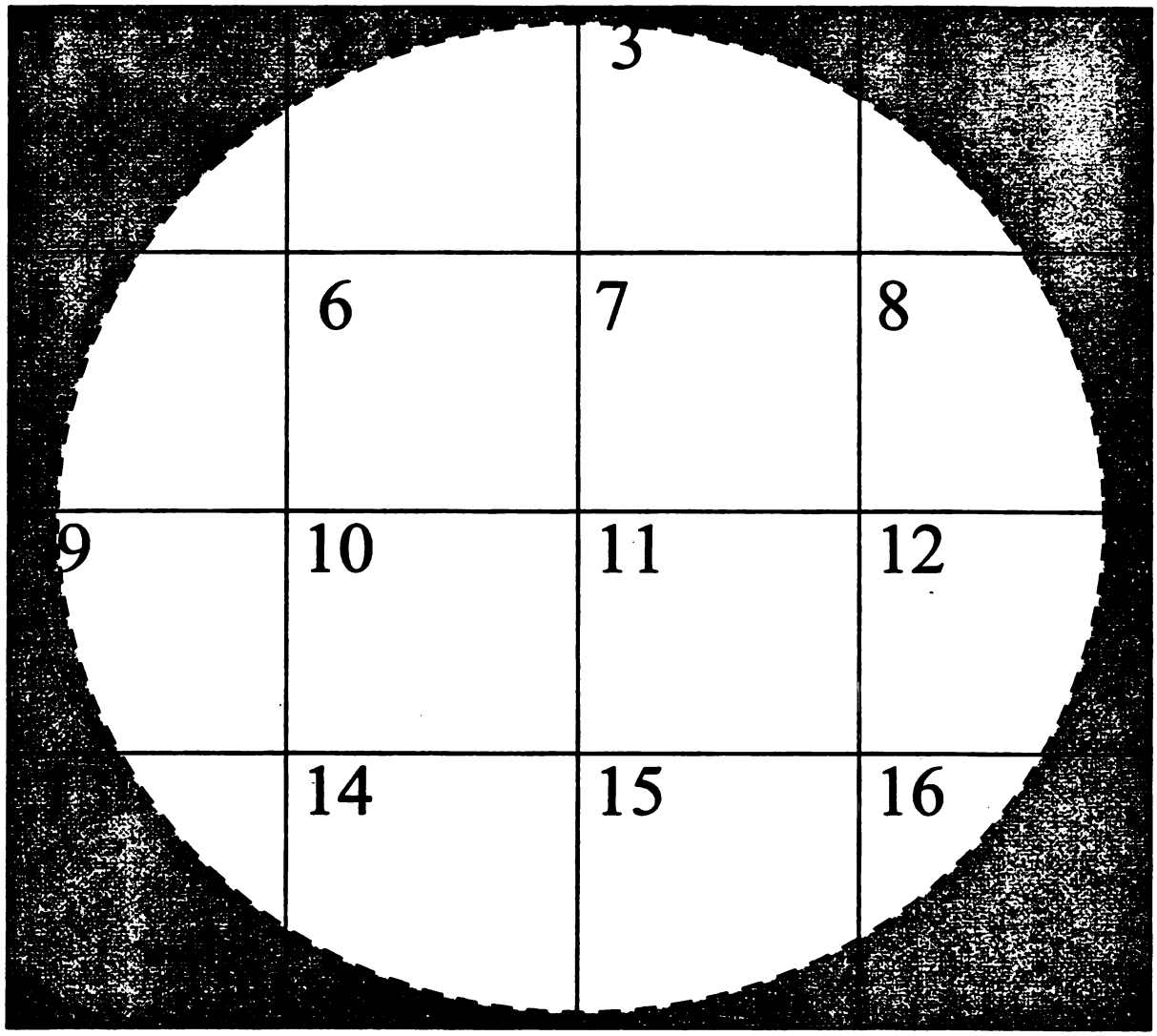
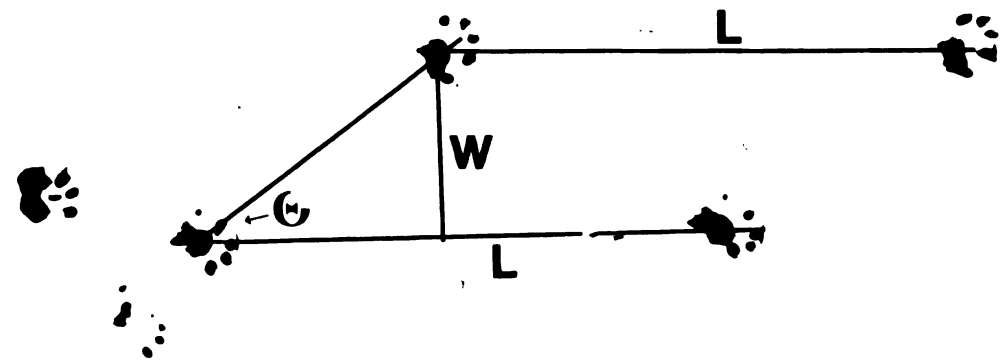


Figure 2: Open-Field Test Diagram



L = stride length, W = stride width, θ = stride angle

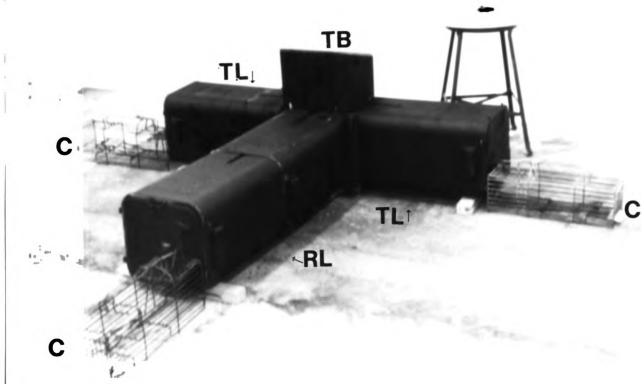
Figure 3: Gait Measurement Test (footprints)

T-Maze Test

The T-maze used in this study was made out of five plastic containers measuring 30.5 cm L x 30.5 cm W x 56 cm H (Figure 4). One container was designated the "turn box", placed upright, and a half-oval hole (11.5 cm W X 10.5 cm H) cut into three of the four sides. Two containers were designated the "turn legs," placed on their sides, and a half-oval hole cut in each of them where they adjoined the turn box at right angles. The remaining two containers were designated the "release leg." The bottom was cut out of one, which was then fitted into the other container. They were placed on their sides and a half-oval hole cut in the end that adjoined the turn box at a right angle. The open ends of the legs were fitted with lids. Circular holes (11 cm dia.) were cut into the lids. The containers and lids were painted black on the exterior surfaces. The maze was held together with screws attaching the containers to 5.08 x 5.08 cm (2 x 2 inch) wooden supports.

Three "catchcages" were used to release and catch the mink. They abutted the opening of each leg and were held in place with elastic "bungee" cords. The doors of the cages were either locked closed with a clip or held open by a trap mechanism. A 5 cm X 9.5 cm piece of glassboard was attached to the cage floor at the end opposite the door. Food, as bait, was placed on the glassboard as needed. The turn box was covered with a clear piece of plexiglass so the person recording could observe the mink's movements.

The T-maze testing began July 15, 1996. Table 2 shows the day-by-day protocol. Only the kits selected for the forelimb grip strength test were used for the T-maze testing. There was an eight-day acclimation period. The kits were fed in crocks inside their home cages during the first three days rather than on the cagetop so that individual feed



C= catchcage, RL = release leg, TB = turn box, TL = turn leg

Figure 4: T-Maze Test Apparatus

Table 2: T-Maze Testing Schedule

	Day	Protocol
Acclimation phase	1	Cages unbaited, locked open; feed *ad libitum*, measure intake
	2	Cages unbaited, locked open; feed *ad libitum*, measure intake
	3	Cages baited, locked open; feed *ad libitum*, measure intake
	4	Cages baited, locked open; restrict feed
	5	Record body weight; cages baited, locked open; restrict feed
	6	Cages baited, locked open; restrict feed
	7	Cages baited, rigged to shut; restrict feed
	8	Record body weight; cages baited, rigged to shut; restrict feed (adjusted amount)
Training phase	9	Select leg; begin training; restrict feed
	10	Continue training; restrict feed
	11	Continue training; restrict feed
	12	Continue training; restrict feed
	13	Continue training; feed *ad libitum*
Rest phase	14	Feed *ad libitum*
	15	Feed *ad libitum*
Challenge phase	16	Record body weight; test for leg trained; challenge; restrict feed (adjusted amount)
	17	Test for last leg; challenge; restrict feed
	18	Test for last leg; challenge; restrict feed
	19	Test for last leg; challenge; feed *ad libitum*

consumption could be measured. On the first two days, each kit was allowed to roam freely inside the maze and the catchcages for five minutes. The doors to the cages were secured open so they would not trip closed, and no bait was placed inside the cages.

On Days 3 and 4 of the acclimation period, each kit was allowed to roam freely inside the maze and the catchcages for five minutes. The doors to the cages were secured open so they would not trip closed. Bait was placed on the glassboard in each catchcage and was replenished if the mink ate the food. On Day 4, after its session in the maze, each kit received 75% of its average feed consumption based on the second and third days' crock feedings.

On Day 5 of the acclimation period, each kit was weighed prior to its session in the maze. This was done to determine what percent of body weight each mink was being fed so that the feed amount could be adjusted as the kits grew. On Days 5 and 6 of the acclimation period, the acclimation protocol was the same as for Days 3 and 4, with each kit receiving the same amount of food it had received on Day 4.

On Days 7 and 8 of the acclimation period each kit was allowed to roam inside the maze for five minutes. The catchcages were baited and their doors were rigged to shut behind the mink when it entered the cage. After the mink was caught in the cage, the door was opened manually and then reset after the mink exited. The side of the maze the mink was caught from was recorded each time to determine if there was a turn preference. On Day 7, each kit was fed the amount of food it had received on Day 4. On Day 8 of the acclimation period, each kit was weighed prior to its session in the maze. The amount of feed was then adjusted to compensate for body weight change.

The acclimation period was followed by a five-day training period. A turn

direction for each mink was determined by coin toss or, if the mink had shown a turn preference, by response reversal. On each day of training, the kit was allowed to enter the maze at the release leg. The door of the release cage was then held closed by the handler. If, at the turn box, the mink entered the "correct" leg, it would find an open catchcage with food in it at the end of the leg. If the mink entered the incorrect leg or re-entered the release leg, it would find a closed catchcage with food in it. Whenever the mink was caught in the correct cage, that cage was moved to the release leg, the empty cage was rigged open and placed at the end of the correct turn leg, and the mink was allowed to enter the maze again. Each mink received five minutes of training per day. Records were kept on incorrect turns, correct turns, catches, whether the mink ate the food, and occurrences of biting or scratching at the closed door of an incorrect cage, jumping on the walls of the turn box, and vocalizing by the mink.

After their sessions on Day 13 (Day 5 of the training period) and for the next 2 days, the mink were fed *ad libitum* and were not handled. During this time, the training records were reviewed and the kits divided into "trained" and "not trained" groups. The "trained" kits were those that had a maximum number of catches and a minimum number of errors. The "not trained" kits were removed from the study.

On July 29, 1996, the "trained" kits were allowed to enter the maze individually and the time from release to capture from the correct leg was recorded. Then the kit was challenged by changing its correct turn leg to the opposite leg. The mink was allowed to roam the maze for five minutes. If it was caught in the new leg, it was re-released as described in the training protocol. If the kit completed 10 consecutive correct (error-less) runs, the turn direction was switched again. Records were kept as described in the training

protocol.

For the following three days, the kits were challenged in the same manner, first being tested in the direction they had last turned correctly the previous day and being timed to determine latency. Latency in the T-maze was defined as the time from when the kit was released into the maze to when it was first caught in a catchcage. During the four days of challenging, the kits were fed a restricted amount of feed based on body weights taken on the first day of the challenge. After the fourth day of challenging, the kits were fed *ad libitum* and the testing was finished.

Stereotypic Behavior Test

Beginning July 20, 1996, the kits selected for the forelimb grip strength test were observed daily for signs of stereotypic behavior. Any behavioral patterns fitting the definition of "stereotypic" as previously described were recorded.

Statistical Analysis

Data collected on the dams were not subjected to statistical testing beyond mean determinations. Kit-testing data were analyzed using statistical software (SAS Institute Inc., 1990).

Kit body weight data, by gender, were tested for treatment effect with a one-way analysis of variance (ANOVA) and compared between treatment groups using Dunnett's test. Eye opening test data were also tested with a one-way ANOVA.

Data from the righting ability and forelimb grip strength tests were arranged in contingency tables and analyzed using the Chi-square test. Tail-pinch test data were also

analyzed with the Chi-square test.

Position in the open field was determined to be either along the perimeter or closer to the center ("interior"). Percent of total time between these two areas was analyzed using the Likelihood Ratio test of a logistic regression analysis. The open-field test data then were divided into percent of total movement by minute subgroups and analyzed using Dunnett's test and Student's t-test. Emotionality scores (occurrences of voiding, vocalizing, jumping, or scratching) were not tested statistically.

Gait measurement test data were subjected to various statistical tests (Likelihood Ration test of a logistic regression analysis, Dunnett's test, Chi-square test) to try to determine if there were any correlations between treatment, sex, weight, and length, width, and angle of stride.

T-maze data were not tested due to the small number of animals trained.

Significant differences were based on $p \leq 0.05$.

RESULTS

Diet Analysis

The results of the proximate, PCB, and mercury analyses of the experimental diets are listed in Table 3. Because the composition of Diet C was identical to that of Diet A except for the addition of methylmercuric chloride, it was assumed that the two diets would be identical nutritionally and in PCB content. Therefore, a sample for Diet C was not submitted for proximate or PCB analysis.

The nutrient values for Diets A and B did not differ by more than 1.19% or 6 ppm except in the zinc concentration. Diet B had nearly one third more zinc than Diet A. Because the primary difference between the ingredients of the two diets was in the fish, it is assumed that the carp was responsible for the additional zinc. All nutritional parameters of the experimental diets exceeded the minimum requirements for pregnant or growing mink (National Research Council, 1982).

Dam Health

Body weights and reproduction data for the dams are shown in Table 4. One adult mink each from Diet A and Diet B died close to weaning. These animals were not subjected to necropsy. Due to their very thin body condition and decreased appetite, it was assumed they were affected by nursing sickness, a condition seen in late lactation in mink (Schneider and Hunter, 1993b).

One female on the control diet refused to accept a male during the breeding season. Occasionally, female mink will display physical signs of estrus but are unwilling to mate.

Table 3: Diet Analyses

	Diet A	Diet B	Diet C
Nutrient analysis (dry matter basis)[1, 2, 3]			
Fat, %	22.20	21.50	
Crude protein, %	39.69	38.50	
Crude fiber, %	3.55	3.25	
Total digestible nutrients, %	94.40	94.36	
Calcium, %	2.40	2.45	
Phosphorus, %	1.59	1.64	
Potassium, %	0.84	0.85	
Magnesium, %	0.22	0.20	
Sodium, %	0.66	0.60	
Iron, ppm	296	290	
Manganese, ppm	50	48	
Copper, ppm	21	25	
Zinc, ppm	95	122	
Total PCB concentration, ppm[4, 5]	ND[6]	0.883	
Total mercury concentration, ppm[7]	<0.1[8]	<0.1	0.325

[1]Proximate analysis of diets by Litchfield Analytical Services, Litchfield, MI.
[2]Dry matter of Diet A was 46.5%, Diet B was 42.33%.
[3]Diet C was assumed to be nutritionally identical to Diet A and was not analyzed for nutrient or PCB content.
[4]PCB analysis by Michigan State University Animal Health Diagnostic Laboratory, E. Lansing, MI. Two samples of Diet B were submitted on different dates. Value shown for Diet B is the mean of the two analyses (0.746 and 1.02 ppm total PCBs).
[5]Test results reported on fat weight basis. Fat recovery for Diet A was 10.7%, Diet B was 9.6% for the first sample, 9.42% for the second sample.
[6]Not detected at 0.025 ppm.
[7]Mercury analysis by Fibertec Environmental Services, Holt, MI. Two samples of each diet were submitted on different dates. Value shown for Diet C is the mean of the two analyses (0.4 and 0.25 ppm total mercury).
[8]Minimum detection limit was 0.1 ppm.

Table 4: Dam Body Weight and Reproduction Data

Table 4: Dam Body Weight and Reproduction Data			
	Diet A	Diet B	Diet C
Body weight means, g ± SE (n)			
Six weeks prior to breeding (start of exposure)	1182 ± 34.6 (12)	1132 ± 27.4 (12)	1144 ± 50.6 (12)
Four weeks prior to breeding	1138 ± 39.3 (12)	1043 ± 27.9 (12)	1104 ± 44.7 (12)
Beginning of breeding period	1153 ± 40.2 (12)	1051 ± 32.6 (12)	1116 ± 46.9 (12)
At whelping	1304 ± 56.5 (9)	1156 ± 39.4 (11)	1255 ± 49.7 (11)
At three weeks postpartum	1151 ± 59.9 (8)	1089 ± 29.4 (10)	1072 ± 44.4 (9)
At six weeks postpartum (litter weaned)	946 ± 45.6 (8)	903 ± 48.4 (10)	917 ± 50.4 (9)
Reproduction records			
Avg. number attempted matings/female, ± SE	5.8 ± 0.5	4.5 ± 0.3	5.2 ± 0.4
Number females successfully mated	11	12	12
Avg. number matings/female successfully mated, ± SE	2.2 ± 0.3	3.1 ± 0.3	2.8 ± 0.3
Number females whelping (% of females mated)	9 (82.8)	11 (91.7)	11 (91.7)
Avg. gestation length (days), ± SE	45.9 ± 1.3	45.1 ± 0.8	46.1 ± 0.5

The gestation length for PCB-exposed dams was almost one full day shorter than the control group but falls within the normal range for mink of 40-75 days (Calabrese *et al.*, 1992).

Kit Health and Growth

Both treatment groups had fewer kits alive at birth than did the control, though less than one kit difference each (Table 5). There was up to three times greater mortality at birth in the treated litters. This may indicate a fetal environment deficient in nutritional or hormonal requirements or the presence of a toxic chemical. No physical deformities were noted in the stillborn kits. The mortality rate between litters began to even out by three weeks postpartum and eventually became greater in the control litters than in the treated litters. This is not to suggest that the treated diets were beneficial to the kits' survival, however, as the litter sizes were reduced by about one third.

In the control group, one litter died before two weeks of age, two litters lost young to a staphylococcal infection, four out of five chilled newborn kits may have died by one week of age, and others may have died due to lack of maternal care. In Diet B, the PCB group, one litter died by three weeks of age, two litters lost young to a staphylococcal infection, and other kits may have died due to lack of maternal care. In Diet C, the MeHg group, two litters died by one week of age and one litter lost young to a staphylococcal infection.

Three newborn kits in one Diet B litter each suffered nonlethal amputations of a limb. While the kits survived the injuries and lived to the end of the study, they were not used as test subjects. Excelsior bedding can wrap around newborn kits' bodies or limbs and is strong

Table 5: Litter Size, Kit Mortality, and Kit Body Weights

	Age (wks)	No.	Diet A	No.	Diet B	No.	Diet C
Avg. litter size (live kits)	0 (birth)		6.0		5.3		5.7
Kit mortality, %	0 (birth)		3.6		9.4		10.8
	3		26.8		26.6		30.8
	6		37.5		35.9		32.3
Body weights (g ± SE)							
Males	0 (birth)	24	10.17 ± 0.49	28	10.59 ± 0.49	33	9.88 ± 0.43
	1	16	28.43 ± 2.05	24	28.47 ± 1.79	20	31.87 ± 1.97
	2	15	69.54 ± 4.09	23	64.54 ± 3.42	20	71.51 ± 3.74
	3	15	110.30 ± 5.46	22	106.18 ± 4.76	20	116.92 ± 4.97
	4	15	152.88 ± 9.82	21	149.74 ± 8.62	20	161.30 ± 8.94
	5	13	198.83 ± 14.89	21	186.12 ± 12.55	19	219.25 ± 13.16
	6	13	283.67 ± 22.73	19	262.95 ± 19.10	19	318.45 ± 19.82
Females	0 (birth)	30	9.40 ± 0.47	27	9.41 ± 0.44	30	9.01 ± 0.44
	1	28	26.56 ± 1.96	24	25.47 ± 1.80	25	29.60 ± 1.95
	2	26	62.08 ± 3.95	23	55.33 ± 3.45	25	65.46 ± 3.72
	3	26	98.92 ± 5.24	22	92.58 ± 4.77	25	105.03 ± 4.93
	4	26	138.21 ± 9.42	22	128.09 ± 8.57	25	150.17 ± 8.86
	5	24	164.55 ± 13.75	22	155.71 ± 12.47	25	193.89 ± 12.89
	6	22	225.08 ± 20.75	19	218.37 ± 19.25	25	279.73 ± 19.33

enough to cut through the thin, hairless skin.

No significant differences were found between the body weights of the control kits and those of the treated kits of either sex.

Righting Ability Test

The righting ability data are tabulated in Table 6. The righting times were categorized to simplify analysis. The majority of kits trying to right themselves could do so within five seconds, Category 1. Category 2 included kits with minor difficulties righting themselves by 10 seconds after release. Category 3, 10 to 30 seconds, was never occupied by a kit older than one week of age. Only one kit, from Diet B, took longer than 30 seconds to right itself at one week of age. No kits older than one week of age exceeded the time limit.

In this study, treatment did not affect a kit's ability to right itself. By three weeks of age, the kits' coordination and alertness were such that continued testing was considered unnecessary. The "not trying" ("n") category was included as an attempt to detect a kit's indifference to the situation. "Not trying" kits whined during the test but did not openly vocalize or flail their legs like those that did try. At one week of age, three kits in Diet A group, six in Diet B group, and two in Diet C group were scored as "not trying." At this age, at least half of the kits in each group were asleep through the test and did not awaken when handled and manipulated gently. At two weeks of age, the percentage of kits sleeping though the test in either treated group was nearly double that in the control group. Statistical analysis showed no difference between "trying" kits, "not trying" kits, and "sleeping" kits.

Table 6: Righting Ability Data[1]

	Category[2]	Diet A	Diet B	Diet C
At birth	No.	54	55	63
	1	79.63	80.00	84.13
	2	3.70	10.91	7.94
	3	11.11	3.64	1.59
	+	3.70	5.45	4.76
	n	1.85	0.00	1.59
	s	0.00	0.00	0.00
One week old	No.	44	47	45
	1	25.00	21.28	31.11
	2	11.36	4.26	13.33
	3	2.27	4.26	0.00
	+	0.00	2.13	0.00
	n	6.82	12.77	4.44
	s	54.55	55.32	51.11
Two weeks old	No.	41	46	45
	1	87.80	69.57	75.56
	2	0.00	2.17	4.44
	3	0.00	0.00	0.00
	+	0.00	0.00	0.00
	n	0.00	2.17	0.00
	s	12.20	26.09	20.00
Three weeks old	No.	41	44	45
	1	97.56	93.18	95.56
	2	2.44	0.00	0.00
	3	0.00	0.00	0.00
	+	0.00	0.00	0.00
	n	0.00	0.00	0.00
	s	0.00	6.82	4.44

[1]Percent of kits within each category.
[2]Categories defined as:
1 = less than 5 seconds
2 = greater than or equal to 5 seconds but less than 10 seconds
3 = greater than or equal to 10 seconds but less than 30 seconds
+ = greater than or equal to 30 seconds
n = conscious but not trying
s = sleeping

Tail-Pinch Response Test

The tail-pinch response test data are presented in Table 7. In this study, the male kits in the treated litters were more reactive, though not significantly, than the male kits in the control litters at both testing ages. The female kits in the treated litters were more reactive at birth than the control cohort but less reactive at three weeks, again not significantly.

Eye Opening Test

The eye opening test data are presented in Table 8. These data indicate the average earliest age and not the average litter age nor the latest postnatal day of eye opening. While PCB-treated kits were very similar to control kits in average age of eye-opening, the range was wider for Diet B. The data indicate that MeHg-treated kits opened their eyes sooner than untreated kits, as evidenced by the lower average age and lower upper limit of the range. However, no statistically significant differences were found.

Forelimb Grip Strength Test

The forelimb grip strength test data are presented in Table 9. The release times were categorized to simplify analysis. There were no statistically significant differences between the groups.

Category 1, gripping for less than 10 seconds, was chosen as an index for kits with minimal clinging ability or weak kits. Progressively stronger kits scored in an appropriately higher category. At least half of the kits could maintain a grip on the rod for 30 seconds. Male kits tended to retain their grip on the rod for a longer period than female kits. PCB- or

Table 7: Tail Pinch Score (Means ± SE)[1]

	No.	Diet A	No.	Diet B	No.	Diet C
Male						
At birth	24	1.79 ± 0.19	28	2.04 ± 0.17	33	1.82 ± 0.13
At three weeks	15	0.80 ± 0.20	22	1.00 ± 0.17	20	1.47 ± 0.16
Female						
At birth	30	1.90 ± 0.14	27	2.07 ± 0.13	30	2.17 ± 0.11
At three weeks	26	1.12 ± 0.17	22	0.86 ± 0.12	25	0.75 ± 0.16

[1]Score ranges from 0 to 3 with 0 being least severe (no response) and 3 being most severe (all responses, see text for descriptions). It is assumed reaction types are equal in severity.

Table 8: Average Age of First Kit in Litter to Open Eye(s).

	Diet A	Diet B	Diet C
No. (litters)	8	10	9
Age (days; mean ± SE)	28.4 ± 1.2	28.0 ± 1.1	26.8 ± 0.7
Range	23-33	22-35	23-30

Table 9: Forelimb Grip Test Data[1]

	Category[2]	Diet A	Diet B	Diet C
Male	No.	5	8	8
	1	0.00	0.00	0.00
	2	20.00	0.00	0.00
	3	20.00	12.50	50.00
	4	60.00	87.50	50.00
Female	No.	7	8	9
	1	0.00	12.50	0.00
	2	28.57	25.00	11.11
	3	14.29	12.50	66.67
	4	57.14	50.00	22.22

[1]Percent of kits within each category.
[2]Categories defined as:
1 = less than 10 seconds
2 = greater than or equal to 10 seconds but less than 30 seconds
3 = greater than or equal to 30 seconds but less than or equal to 60 seconds
4 = greater than 60 seconds.

MeHg-treatment seemed to "improve" (move to a higher category) the scores of the treated male kits compared to controls. The PCB-treated females had a lower score than control females.

Open-Field Test

The open-field test data are summarized in Tables 10A, 10B, and 10C, corresponding to data gathered at six, seven, and eight weeks of age, respectively. One female kit in the control group contracted a systemic staphylococcal infection after six weeks of age and was euthanized.

As six-week-old kits, the mink displayed greater overall hesitation. As they matured, the kits as a whole moved more readily, often following the wall, around the open-field. Initially the PCB group (B) changed grid position less frequently but then became more active than controls by eight weeks of age. The male kits in the MeHg group (C) did not differ greatly from controls in ambulatory activity. The female kits in group C, however, were consistently more active than controls. The relatively close values between each minute for movement scores indicate that the kits did not experience any initial panic, which would have resulted in an immediate surge in activity, nor did they suppress exploration in order to adapt to the situation, which would have resulted in an increase in movement over time. Statistical analysis of position and movement data revealed no significant differences.

At least one kit of each gender per treatment voided during the test at all ages, except for seven-week-old females in Diet C. Only one kit, a seven-week-old control male, vocalized during the test. Incidences of jumping or scratching increased over time but no

Table 10A: Open-Field Test Data for 6-Week-Old Kits (Means)

	Diet A	Diet B	Diet C
Males			
No.	5	8	8
Position, % of total time			
Perimeter	39.17	56.25	41.04
Interior	60.83	43.75	58.96
Grid changes ± SE	35.2 ± 4.6	26.5 ± 3.6	31.0 ± 6.7
Movement, % of total movement			
First minute	38.64	37.32	36.99
Second minute	35.80	31.10	33.33
Third minute	25.57	31.58	29.67
Emotionality, % of kits			
Voiding	40.00	37.50	25.00
Vocalization	0.00	0.00	0.00
Jumping or scratching	0.00	0.00	0.00
Females			
No.	7	8	9
Position, % of total time			
Perimeter	46.55	41.25	60.83
Interior	53.45	58.75	39.17
Grid changes ± SE	33.1 ± 7.5	22.1 ± 4.3	36.8 ± 5.8
Movement, % of total movement			
First minute	36.40	32.97	32.00
Second minute	32.46	35.68	31.38
Third minute	31.14	31.35	36.62
Emotionality, % of kits			
Voiding	14.29	37.50	44.44
Vocalization	0.00	0.00	0.00
Jumping or scratching	0.00	0.00	11.11

Table 10B: Open-Field Test Data for 7-Week-Old Kits (Means)			
	Diet A	Diet B	Diet C
Males			
No.	5	8	8
Position, % of total time			
Perimeter	82.17	84.48	84.06
Interior	17.83	15.52	15.94
Grid changes ± SE	43.4 ± 5.2	48.1 ± 7.3	48.5 ± 3.9
Movement, % of total movement			
First minute	29.49	31.40	31.07
Second minute	34.10	32.72	33.16
Third minute	36.41	35.88	35.77
Emotionality, % of kits			
Voiding	40.00	37.50	12.50
Vocalization	20.00	0.00	0.00
Jumping or scratching	20.00	37.50	0.00
Females			
No.	6	8	9
Position, % of total time			
Perimeter	78.06	87.29	88.89
Interior	21.94	12.71	11.11
Grid changes ± SE	50.2 ± 6.5	44.3 ± 4.1	57.2 ± 4.5
Movement, % of total movement			
First minute	31.63	33.62	31.33
Second minute	35.37	33.91	40.76
Third minute	32.99	32.47	27.91
Emotionality, % of kits			
Voiding	50.00	12.50	0.00
Vocalization	0.00	0.00	0.00
Jumping or scratching	0.00	12.50	0.00

Table 10C: Open-Field Test Data for 8-Week-Old Kits (Means)

	Diet A	Diet B	Diet C
Males			
No.	5	8	8
Position, % of total time			
Perimeter	85.33	83.85	83.85
Interior	14.67	16.15	16.15
Grid changes ± SE	62.2 ± 5.0	68.4 ± 6.1	62.1 ± 2.9
Movement, % of total movement			
First minute	36.07	31.18	35.66
Second minute	30.16	36.16	34.43
Third minute	33.77	32.66	29.92
Emotionality, % of kits			
Voiding	20.00	50.00	62.50
Vocalization	0.00	0.00	0.00
Jumping or scratching	20.00	12.50	12.50
Females			
No.	6	8	9
Position, % of total time			
Perimeter	89.17	86.25	87.87
Interior	10.83	13.75	12.13
Grid changes ± SE	59.0 ± 4.4	61.8 ± 2.7	70.7 ± 3.5
Movement, % of total movement			
First minute	38.40	34.78	34.83
Second minute	33.52	32.92	32.91
Third minute	28.08	32.30	32.26
Emotionality, % of kits			
Voiding	16.67	12.50	55.56
Vocalization	0.00	0.00	0.00
Jumping or scratching	16.67	0.00	11.11

trend was evident.

Gait Measurement Test

The ranges for the data for the gait measurement test are listed in Table 11. The variability in the data collected for this behavior precluded determining a correlation between the measured parameters.

At six weeks of age, four male kits and three female kits in the control group were not active, two male kits and two female kits in the PCB group were not active, and one female kit in the MeHg group was not active. At seven weeks of age, only one kit, a female from the MeHg group, ran during the test, nullifying her data. At eight weeks of age, that same kit and one male from the PCB group ran during the test.

The data in this study indicate that as the kits matured, their stride length tended to increase. The stride width tended to decrease as weight increased, perhaps due to a higher center of gravity and improved coordination. The average sine of the angle, θ, formed by the placement of the two hindfeet relative to the direction of movement, decreased with age, except for the female kits in Diet C from six to seven weeks of age, indicating a more acute angle or longer stride side to side. Except at six weeks of age, the male kits had a greater mean sine of θ than the female kits within the same treatment.

T-maze Test

The T-maze test data are shown in Table 12. In this study, 24 out of 44 animals were not considered trainable. Of the 24, 22 did indeed get caught in the appropriate cage,

Table 11: Gait-Measurement Test Data

	Diet A	Diet B	Diet C
Males			
Weight ranges, g (n)			
Six weeks	359 (1)	174-351 (6)	209-404 (8)
Seven weeks	397-513 (5)	309-516 (8)	422-627 (7)[1]
Eight weeks	559-713 (5)	446-678 (7)	532-820 (8)
Stride length ranges, cm (n)			
Six weeks	13.4 (1)	7.6-16.4 (6)	8.7-15.6 (8)
Seven weeks	11.9-17.7 (5)	9.3-16.0 (8)	13.2-18.8 (8)
Eight weeks	16.0-20.9 (5)	12.7-19.3 (7)	15.2-26.1 (8)
Stride width ranges, cm (n)			
Six weeks	8.0 (1)	3.4-6.4 (6)	3.4-7.3 (8)
Seven weeks	4.5-8.1 (5)	4.2-7.4 (8)	4.2-8.3 (8)
Eight weeks	3.8-7.7 (5)	4.6-7.7 (7)	4.3-6.8 (8)
Mean sine $\theta \pm$ SE (n)[2]			
Six weeks	0.72 (1)	0.72 ± 0.050 (6)	0.68 ± 0.027 (8)
Seven weeks	0.63 ± 0.081 (5)	0.64 ± 0.032 (8)	0.62 ± 0.042 (8)
Eight weeks	0.63 ± 0.071(5)	0.58 ± 0.035 (7)	0.46 ± 0.059 (8)
Females			
Weight ranges, g (n)			
Six weeks	187-290 (4)	173-311 (6)	199-309 (7)
Seven weeks	280-478 (6)	255-445 (8)	367-468 (7)[1]
Eight weeks	404-595 (6)	353-560 (8)	470 - 580 (8)
Stride length ranges, cm (n)			
Six weeks	9.5-16.0 (4)	6.8-11.9 (6)	9.8-13.6 (7)
Seven weeks	11.8-15.8 (6)	8.2-14.4 (8)	12.1-18.1 (8)
Eight weeks	15.8-19.2 (6)	12.4-20.9 (8)	14.3-26.4 (8)
Stride width ranges, cm (n)			
Six weeks	4.6-5.6 (4)	3.8-6.2 (6)	3.4-6.2 (7)
Seven weeks	3.7-5.6 (6)	3.2-6.4 (8)	3.6-6.5 (8)
Eight weeks	2.3-8.0 (6)	2.8-7.0 (8)	2.0-7.4 (8)
Mean sine $\theta \pm$ SE (n)[2]			
Six weeks	0.73 ± 0.040 (4)	0.69 ± 0.054 (6)	0.59 ± 0.023 (7)
Seven weeks	0.59 ± 0.045 (6)	0.54 ± 0.037 (8)	0.62 ± 0.025 (8)
Eight weeks	0.55 ± 0.073 (6)	0.53 ± 0.047 (8)	0.40 ± 0.050 (8)

[1]Weight of one animal not recorded.
[2]θ is the angle formed by the placement of the two hindfeet relative to the direction of movement.

Table 12: T-Maze Test Data

	Diet A	Diet B	Diet C
Male			
Training period			
Mean age, days ± SE	84.0 ± 0.9	81.0 ± 1.4	81.3 ± 1.4
No. with preferred turn	1	2	3
No. attempted to train	5	8	8
No. trained successfully (%)	3 (60)	4 (50)	6 (75)
Testing period			
Mean age, days ± SE	84.3 ± 1.2	82.5 ± 0.8	80.8 ± 1.8
Latency range, sec	2.5-12.1	6.6-98.8	2.6-55.0
No. with successful reverse (%)	3(100)	4(100)	6(100)
No. biting	1	2	4
No. scratching	3	4	5
No. jumping	2	3	0
Female			
Training period			
Mean age, days ± SE	83.8 ± 0.7	81.9 ± 1.6	81.1 ± 0.9
No. with preferred turn	4	2	3
No. attempted to train	6	8	9
No. trained successfully (%)	2 (33)	3 (38)	2 (22)
Testing period			
Mean age, days ± SE	83.0 ± 0.7	84.3 ± 1.4	85.0 ± 2.8
Latency range, sec	15.1-76.3	8.1-62.3	1.6-20.5
No. with successful reverse (%)	1(50)	3(100)	2(100)
No. biting	2	3	1
No. scratching	2	3	2
No. jumping	1	1	1

however their training records revealed that the kits were inconsistent in the trials and did not get caught the number of times required to be considered successfully trained. Similar results were seen in practice trials run the previous year on different, untreated mink kits (data not shown). In this study, these results are not related to treatment but are related to gender, with females less likely to be trained than males. Also, those animals with a preferred leg to enter during acclimation proved more likely to be trained than those without a preference.

The data suggest that untreated male mink kits have shorter latencies than untreated female kits. Of the mink considered trained in the T-maze test, the PCB-treated male kits seemed to be slower to complete the task than control males. Four of the six MeHg-treated male kits had shorter average latencies than the control males, but the other two kits showed greater hesitation. The female kits of either treatment group tended to have shorter latencies than the control females.

Biting and scratching in the T-maze were interpreted as signs of frustration. Females displayed more instances of frustration than males. However, there was no statistically significant treatment effect.

Jumping was interpreted as distractibility in the animal. This behavior was noticed only in the turn box, the only area in the maze that had a higher and a transparent ceiling. It is possible a mink could have been distracted by the person recording, who must look into the turn box from above in order to record the animal's movement. The males in the MeHg-treated group never jumped when they were in the maze. Viewed in a positive light, this could mean that they were devoted to the task of going to the cage to receive their reward.

Alternatively, this group of animals could have been stuck in a behavior pattern, out of which they would emerge only by being severely distracted or startled.

Stereotypic Behavior Test

Only one animal out of 44 displayed any stereotypic activity, a female from Diet C, the MeHg treatment. The actions displayed were a circular rearing against the back of the cage with the posterior portion of the body remaining still.

DISCUSSION

Diet Analysis

PCB concentrations in fish from three major Michigan rivers ranged from 0.03 to 6.0 ppm and were 0.16 to 6.0 ppm in carp. The range of mercury in fish from those same rivers was 0.05 to 0.73 ppm (Giesy *et al.*, 1994b). Assuming that these concentrations are representative throughout mink habitat and that a wild mink's diet consists of about 30% fish (Heaton *et al.*, 1995a), wild mink would be exposed to 0.009 to 1.8 ppm PCBs and 0.015 to 0.219 ppm total mercury just through consumption of fish. Other sources for these contaminants would be frogs, crustaceans, insects, small birds and young waterfowl, small mammals, and turtle or bird eggs. Lead and cadmium have been found in muskrat tissue (Erickson and Linzey, 1983), a prey species of the mink, suggesting another source of heavy metal exposure for mink. The concentrations of PCBs or mercury in the experimental diets in this study fell within or above, respectively, the ranges predicted for natural exposure.

Aulerich *et al.* (1991) fed adult and growing mink diets containing up to 1,500 ppm supplemental zinc as zinc sulfate for 144 days and found no detrimental effect on the animals' health. Bleavins *et al.* (1983) fed gestating and lactating mink and their unweaned litters 1000 ppm supplemental zinc as zinc sulfate. They observed no signs of toxicity in the dams. However, the kits from the zinc-treated dams displayed signs of copper deficiency while they were still nursing and exhibited profound, but not permanent, immunosuppression. Since the concentration of zinc in Diet B in this study was nearly one order of magnitude less than that seen in the earlier reports, it is assumed that 122 ppm is not a level for concern.

Dam Health

Many behavioral effects exhibited in newborn and adult animals are subtle. Overt neurologic symptoms occur less frequently than deficits or delays. Damage might only be detected when the injured system is challenged (Rodier *et al.*, 1994). Changes in maternal health or hormonal status during gestation or lactation can cause neural effects in the young (Zbinden, 1981; Spear, 1990). Other non-specific effects such as hypoxia, hypothermia, hyperthermia, and changes in nutritional status can dispose the neonate to neurological damage (Spear, 1990). In this study, the body weight means of the dams (Table 4) did not show any notable trends. It is normal for a nursing female to lose a considerable amount of weight during lactation and to weigh less than when she was first bred (Korhonen, 1988; Hansen, 1997). The reproduction records of the dams in this study did not suggest any hormonal changes that could affect willingness to breed or the ability to successfully whelp a litter. It is a routine practice at the MSU Experimental Fur Farm and on commercial mink farms to have females mated "successfully" (sperm checked) two or more times.

Kit Health and Growth

Changes in kit growth and body weight may indicate developmental neurotoxicity (Rodier *et al.*, 1994). Lochry *et al.* (1994) found that some behavioral effects and physical developmental landmarks were associated with body weight. In this study, growth of the kits in the treated groups did not vary significantly from the control (Table 5), as was also found by Wren *et al.* (1987b) for mink exposed to PCBs and MeHg.

The mortality experienced by the litters at three and six weeks was greater than that

calculated for non-research litters whelped the following year (MSU farm records). In 1997, "ranch" litters on the Experimental Fur Farm experienced 12.1, 19.3, and 21.7 percent mortality at birth, three, and six weeks of age, respectively. Mortality at birth for 1996 "ranch" litters was 5.5 percent (MSU farm records). Data were not collected at three and six weeks previous to 1997. Schneider and Hunter (1993a) calculated mortality at birth for over 18,000 mink kits to be 10.6 percent. Wenzel *et al.* (1984) found 15 percent mortality for over 7,500 newborn mink kits. Howell (1979) determined mortality from birth to three weeks of age for mink to range up to 24 percent. Schneider and Hunter (1993a) calculated preweaning mortality to be 20 percent. As shown in Table 5, all diet groups in this study exceeded these "normal" three- and six-week mortality figures. It is possible that the frequency of handling the kits caused the dams anxiety, which in turn would cause them to be more excitable when the kits were taken from or returned to the nestbox, possibly trampling, biting, or neglecting the young.

Losses of kits after birth can be attributed to underweight kits, cannibalism, rearing and maintenance faults, agalactia, extreme litter size, deformities, or unknown causes (Wenzel *et al.*, 1984). In this study, all of the dams were primiparous and lacked experience with raising young. All diet groups experienced a staphylococcal infection in some of the litters. "Ranch" litters also were affected by a staphylococcal infection (MSU farm records). This type of infection has been documented in mink elsewhere (Hunter and Prescott, 1991). It is assumed the dietary treatments did not predispose the kits to the infection since control litters were also affected.

Righting Ability Test

In behavioral teratology research, the primary test for sensory function is a reflex test, the righting ability test being the most frequently used endpoint, followed by auditory startle response (Lochry *et al.*, 1994). It is possible that the higher than expected incidences of "not trying" kits were because the kits were in a state of semi-consciousness rather than not reacting to the test.

If the trend toward sleeping through the test had also been strongly evident at two and three weeks of age, it would suggest that the milk from PCB- or MeHg-treated dams had a narcotic effect. However, that is not seen in these data. If the milk from treated dams had been detrimental to the kits, one would expect that mortality rates for the kits in the treated litters would have increased at a greater rate than that of control (Table 5).

Maternal exposure to up to 10 mg/kg/day of Fenclor 42 by intraperitoneal injection did not affect the righting ability of rat pups (Pantaleoni *et al.*, 1988). However, Thiel *et al.* (1994) found that rats exposed peri- and postnatally to up to 1.0 μg/kg TCDD via subcutaneous injection had an initially higher positive response in the righting ability test. On the third and fourth testing days, the response of the treated rats did not differ from that of the controls. TCDD has been found in carp from Saginaw Bay (Tillitt *et al.*, 1996) and may have been present in Diet B in this study. If TCDD acts to enhance the righting reflex, then the data suggest that any TCDD in Diet B was overcome by the effects of other contaminants, specifically PCBs.

Sobotka *et al.* (1974) found no significant differences in righting ability between treated and control groups of rats exposed *in utero* to up to 2.5 mg/kg methyl mercury

chloride by gavage to the dam during organogenesis.

Tail-Pinch Response Test

The other reflex test used in this study was the tail-pinch response test. This test detects changes in central nervous system excitability such as increased irritability and reactivity.

One theory to explain the different reactions between males and females in this study is that the procedure for the test was flawed. The pinch lacked consistency. Perhaps a mechanical pinch, set at a constant pressure, would yield more reliable results.

Macaques treated orally from birth to about seven years of age with 50 μg/kg/day of methymercuric chloride showed an insensitivity to a pin prick when they were tested at 13 years of age (Rice, 1989). While not exposed *in utero*, the macaques were exposed while the nervous system was still developing. It is possible that mink exposed *in utero* and/or lactationally to MeHg might not exhibit neurotoxic effects in reflex tests until much later in life.

Eye Opening Test

Age at eye opening is an often-used physical developmental landmark. Zbinden (1981) states that gestation length can affect the developmental timetable, however, in this study the difference between the gestation length for the control groups and either of the treated groups was less than one day (Table 4). Because the mink experiences delayed implantation, gestation length can vary from 40 to 75 days (Calabrese *et al.*, 1992). This

variability may skew the age when developmental landmarks are observed.

Perinatal dietary exposure of rats to up to 26 ppm Aroclor 1254 did not affect age at eye opening in the offspring (Overmann *et al.*, 1987). However, three of eight mice exhibiting a spinning syndrome after *in utero* exposure to 32 mg/kg 3,3',4,4'-TECB (via gavage to the dam on gestation days 10 through 16) had not opened both eyes by 65 days of age (Tilson *et al.*, 1979). While one "spinner" experienced stunted growth, there were no significant differences in body weight between treatment groups. Six of eight TECB-spinners were rated as having normal visual placement responses at 35 days of age, but only four of eight were rated normal at 65 days of age. The authors did not indicate whether the depressed response occurred in the mice with delayed eye opening.

In this study, mink exposed *in utero* and lactationally to PCBs opened their eyes almost half a day earlier than controls but the upper limit of the range in age was higher. Aulerich *et al.* (1988) did not find any significant differences in age of eye opening in mink kits treated neonatally with 0.1 μg/kg body weight TCDD (intraperitoneal injection).

When exposed *in utero* on gestation days 6 through 15 to 2.5 mg/kg methyl mercury chloride (gavage to the dam), male rats opened their eyes one day earlier than controls (Sobotka *et al.*, 1974). The authors felt the early eye opening along with an enhanced development of neuromotor coordination reflected a certain degree of compressed central nervous system development. In this study, mink exposed *in utero* and lactationally to MeHg opened their eyes almost one and a half days earlier than the controls. The upper limit of the range in age for the MeHg kits was lower as well. An effect similar to that seen by Sobotka *et al.* (1974) may be responsible for the difference in eye opening age observed with the mink

in this study.

Jonasen (1987) observed the ontogeny of mink kits and found the age of eye opening ranged from 30 to 36 days. In this study, if the kits in a litter had been examined until all had opened their eyes, a clearer indication of the average eye-opening age may have been determined and might have yielded more definitive results.

Forelimb Grip Strength Test

The forelimb grip strength test detects loss of muscular strength and impaired neuromuscular functions. During the test, the subject may fall or it may cling tenaciously (Zbinden, 1981). Duration of suspension may show a nonlinear relation to body weight, such that underweight or small-for-age kits grip the rod for a longer time (Overmann *et al.*, 1979). None of the kits used in the forelimb grip strength test in this study were considered below normal for their age.

When exposed perinatally to up to 26 ppm Aroclor 1254 in the maternal diet, rat pups were not affected in duration of forepaw suspension (Overmann *et al.*, 1987). However, Tilson *et al.* (1979) found TECB-spinners (mice) to have significantly lower forelimb grip strength scores than controls. TECB-nonspinners had lower scores than controls at 65 days of age but the difference was not statistically significant. The authors attributed the neuromuscular dysfunction to muscular weakness. In rats exposed peri- and postnatally to a subcutaneous injection of up to 1.0 μg/kg TCDD, the rate of successfully responding animals was increased in the forelimb grip strength (forelimb-grasp reflex test) test (Thiel *et al.*, 1994). If TCDD acts to enhance the forelimb-grasp reflex and PCBs act to depress it

in mink, then in this study there was a gender-specific response to Diet B, in that the males showed an improved (higher) score over controls while the females had a worse (lower) score than the controls. This may indicate a greater susceptibility to PCB-induced neurotoxicity in female mink exposed *in utero* and lactationally.

In male rats exposed *in utero* (via gavage to the dam) on gestation days 6 through 15 to up to 2.5 mg/kg methyl mercury chloride, the rate of development of the pups' clinging ability was facilitated, more so by the lower doses (0.1 and 0.5 mg/kg) than by the higher dose, although the clinging ability in all treatment groups was significantly greater than controls (Sobotka *et al.*, 1974). In this study, all but one male mink kit exposed *in utero* and lactationally to MeHg clung to the rod for at least 30 seconds. The MeHg-treated female kits showed less variability than control females and showed improvement over controls in that more animals could cling past 30 seconds.

By six weeks of age, mink kits are already displaying secondary sex characteristics. Male kits have larger, more square-shaped heads than female kits and are larger overall (Table 5). The females may be less developed physically in the forequarters than the males, and this may explain why the females, as a group, had shorter grip times than the males.

Macaques tested at 13 years of age, after developmental exposure to 50 μg/kg/day (orally) of methylmercuric chloride from birth to about seven years of age, slipped when climbing the bars in their cages and did not jump from bar to bar (Rice, 1989). It is possible mink exposed *in utero* and/or lactationally to MeHg might show muscular incoordination later in life.

Open-Field Test

The open-field test is designed to help evaluate an animal's emotionality. Interfering factors to consider when using this apparatus are the effects of handling (repeated handling may decrease the emotionality score, whereas handling itself may upset the animal and augment the score) and litter size (competition within a litter might cause the kits to be more lively but less excitable) (Zbinden, 1981; Rodier *et al.*, 1994). A decrease in frequency of urination or defecation by the animal or an increase in latency, that is, fewer grid changes in the allotted time, is an indication of an indifference to a new environment (Zbinden, 1981).

Preconception exposure of the dam and postnatal exposure to up to 50 mg/kg Fenclor 42 (via intraperitoneal injection to the dam) suppressed open-field activity in 14-day-old rats (Pantaleoni *et al.*, 1988). *In utero* exposure did not significantly affect activity. In the same rats at 21 days of age, preconception exposure still caused suppressed activity, whereas test results from postnatally-exposed rats were similar to controls. Lilienthal *et al.* (1990) supplemented the diets of female rats with 30 ppm Clophen A30 and found the offspring to have significantly higher activity levels than animals on the lower treatment level (5 ppm) or control offspring at 22 days of age. At 120 days of age, open-field activity was nearly identical among the three groups. Mice exposed *in utero* and lactationally to up to 82 ppm dietary Aroclor 1254 habituated more slowly to an open field than did the controls, traversing more squares than controls in all time periods after the first period (Storm *et al.*, 1981).

Prenatal exposure on gestation day 7 or 9 to 8 mg/kg dam weight methylmercury dicyandiamide (via intraperitoneal injection to the dam) caused significant differences from controls in rats tested in an open field (Spyker *et al.*, 1972). Specifically, offspring from

treated mothers took a longer time to begin exploration and, when they did, half of the subjects (10 out of 20) took three or more backwards steps during the test period, three of the rats doing so for more than half the test session. Only one of the control rats (out of 19) displayed this behavior. The treated offspring also showed a significantly lower emotionality score in that they voided less frequently than controls during the test. Frequency of rearing and grooming was decreased, though not significantly, in the treated group. Su and Okita (1976) exposed pregnant mice to up to 12 mg/kg methylmercury hydroxide administered subcutaneously in a single dose or in multiple doses. When they tested the offspring in an open field, they found significant decreases from controls in center latency and ambulatory activity in both dose-frequency groups. Mice exposed *in utero* to a single dose groomed themselves and urinated less frequently than controls. Mice exposed *in utero* to multiple doses exhibited less rearing and more backing than controls.

In this study, when the kits were placed in the open-field testing apparatus at six weeks, they were still at an age where motor skills and exploratory behavior were minimal. Up to this age, the kits had relied on the comfort and security of their dam and had little exploratory experience beyond their nestbox and feedplate. Perhaps a longer testing session (five minutes) and beginning the testing at a later age (seven weeks) would provide more definitive data to evaluate ambulatory and exploratory activity.

The voiding score can be misleading. Mink defecate one large stool at one time whereas rodents pass fecal boluses sporadically. When rodents are subjected to an open-field test, the number of times they void are counted. Because the mink kits in this study would urinate or defecate only once during the test, the number of animals that did so were

recorded. Therefore, voiding scores may be inappropriate when testing mink in an open field.

The almost complete lack of vocalization by the kits during this testing phase was unexpected. Rather, since the kit was alone, removed from the security of the litter, vocalizing was expected. The subdued lighting in the testing room may have calmed the kits.

Jumping and scratching can be interpreted as escape behavior. The kits displayed more of this behavior as they matured, however, it was viewed less as an escape behavior and more as a reaction to being distracted by the observer, in the cases of jumping, or as exploratory behavior, due to the posterboard overlaps in the wall's construction, in the cases of scratching.

Gait Measurement Test

Gait measurement can provide insight to an animal's level of confidence and sense of balance. In this study, the kits were tested for gait measurement at the same age as for the open-field test. As discussed previously, at six weeks of age the kits proved to be timid. Their motor skills and exploratory behavior were only just developing as was evidenced by 12 animals being judged "not active," that is, not walking across the paper. At seven weeks of age, inactivity was not a problem. At eight weeks of age, the kits walked quickly away from the handler after being released, sometimes bolting into a run. The records suggest that testing only at seven weeks of age would provide the most accurate gait data.

Although placement of a nestbox was used to encourage the kits to walk a straight line, rarely was the gait that direct. Perhaps releasing the kit into a channel directing it

toward the nestbox would provide footprints that would be easier to measure. Another alternative would be to place the kit on a clear glass surface without painting its feet but rather videotaping its movement from underneath. This would yield more detailed foot placement data.

The offspring of female rats exposed on day 14, 15, 16, or 17 of gestation to up to 125 r of X irradiation had a wider stance and broader angle than control rats (Mullenix *et al.*, 1975). Histologic examination of the brains of the rats showed differences in telencephalic commissures between control and treated groups. In this study, hopping and waddling instances did occur but they were viewed as the kit's response to fear, preparing to bolt, or as a balance compensation in the younger kits. The brains of the mink in this study were not examined histologically.

Prior to full development of walking ability, four- to five-day-old rats display circular locomotory movements called pivoting (Adams, 1986). At this age, forelimb function is better developed than hindlimb function. Pivoting progressively decreases as the hindlimbs strengthen and walking prevails. In this study, similar movement was seen in six-week-old mink kits. They would use their forelimbs as the primary source of locomotion at this age while their hindlimbs still paddled in a walking motion but supported little weight. By seven weeks of age, full walking ability was evident in all kits.

T-Maze Test

"Learning" is the acquisition of knowledge or skill. "Memory" is the capacity for the retention of that which is learned. Failure to perform a learned task can be due to a

disruption in the retention mechanism or the inability to retrieve the information (Pilcher, 1979). Schantz *et al.* (1995) found that female rats exposed *in utero* and via lactation to 2,4,4'-TRCB (up to 32 mg/kg/day), 2,3'4,4',5-PECB (up to 16 mg/kg/day), or 2,2',4,4',5,5'-HCB (up to 64 mg/kg/day) (via gavage to the dam on gestation days 10 through 16) learned a T-maze delayed alteration task more slowly than control females. PCB-exposed male rats did not display this deficit but did tend to have shorter average latencies than control males. Because male rats typically have longer latencies than female rats in maze learning tests, the authors suggested that pre- and postnatal exposure to PCBs shifted male rat behavior toward a female pattern. Perhaps the PCB diet in this study had a feminizing effect in that the latency of the PCB-treated male mink kits was greater than that of control males.

The prefrontal cortex of the brain is the most probable site of damage following developmental exposure to PCBs (Tilson and Harry, 1994). While unique functional units exist in the central nervous system, some areas have reserve capacity (Rodier *et al.*, 1994). However, neurons forced to compensate for damaged neurons might not be able to sustain functioning for the usual life-span, allowing deficits to manifest themselves in mature or aging organisms, as seen by Rice (1989) and Spyker (1975).

Memory circuits involve the hippocampus, amygdala, and thalamus (Manning and Dawkins, 1992). Norepinephrine, serotonin, and dopamine are involved in learning and memory (Pilcher, 1979). Exposure of mink to up to 25 ppm hexachlorobenzene in the feed for 47 weeks resulted in elevated hypothalamic serotonin levels (Bleavins *et al.*, 1984). Hypothalamic dopamine concentrations in the offspring of the 1- and 5-ppm exposed mink were depressed. In ferrets exposed to 250 or 500 ppm hexachlorobenzene for seven weeks,

norepinephrine, serotonin, and dopamine concentrations in the brain were elevated. During the last week of the study, four of the ferrets displayed slight aggressiveness and hyperexcitability (Bleavins *et al.*, 1984). Brain levels of dopamine and norepinephrine were reduced in ring-necked doves fed dichlorodiphenyl dichloroethene (DDE), dieldrin, or Aroclor 1254 (Heinz *et al.*, 1980), which might lead to behavioral aberrations in contaminated birds in the wild. In this study, DDE was detected in Diet B at 0.64 ppm (MSU Animal Health Diagnostic Laboratory, case #1910881), however neurotransmitter concentrations were not measured. In macaques orally exposed to Aroclor 1016, three *ortho*-substituted nonplanar PCBs were detected in the caudate, putamen, substantia nigra, and hypothalamus (Seegal *et al.*, 1990). A decrease in dopamine concentrations was also noticed in those regions in that study. Seegal *et al.* (1991) found that mixtures of PCBs lowered dopamine levels more, additively or synergistically, than did individual congeners.

Thyroid hormones play a role in brain maturation as well as in myelinogenesis, protein and nucleic acid metabolism, and electric activity of the growing brain (McKinney and Waller, 1994). Prenatal exposure via gavage to the dam of up to 1.8 mg/kg 3,3',4,4',5,5'-HCB on gestation day 1 and to 1 mg/kg/day 3,3',4,4'-TECB thereafter resulted in hypothyroidism in the brains of fetal and neonatal rats (Morse *et al.*, 1993). T-4 binding proteins are ideally suited to bind *meta*- and *para*-substituted, dioxin-like PCBs (McKinney and Waller, 1994). However, Seegal *et al.* (1991) argues that lower-chlorinated, *ortho*-substituted congeners may be more neurotoxic than more highly chlorinated ones and that dechlorination processes may increase bioavailability of neurotoxic congeners. Macaque offspring nursing during maternal dietary exposure to PCBs were exposed to the same

congeners as the dams, whereas postexposure nursing exposed the young only to the stored congeners (Schantz *et al.*, 1991), which may have undergone dechlorination. In mink, placental transfer of PCBs is less consequential than lactational transfer (Bleavins *et al.*, 1981).

Also involved in memory and learning as well as in audition, vision, aggression, and neurological syndromes is the cholinergic system (Eriksson, 1988). Adrenocorticotropic hormone (ACTH) affects acquisition and extinction in active and passive conditioned avoidance paradigms and facilitates reversal learning (Pilcher, 1979). *Para*-substituted PCBs and tetrachlorodioxins affect endocrine hormones and vitamin homeostasis (Giesy *et al.*, 1994a). In this study, if ACTH and other hormones had been assayed, perhaps a correlation between learning ability and hormone levels in mink could have been determined.

The offspring of female macaques exposed to 50 μg/kg/day of dietary methylmercury hydroxide displayed attentional problems and were slower than controls to develop object permanence, a stage of the sensorimotor period of cognitive development in primates (Burbacher *et al.*, 1986). In this study, refusal to orient to the maze was not limited to the MeHg-treated kits, nor did treatment affect a kit's learning ability or behavioral plasticity.

Heavy metals cause greater developmental neurotoxicity in postnatal exposure than in prenatal exposure but there is greater sensitivity to lipophilic chemicals with prenatal exposure (Kamrin *et al.*, 1994). Opposite to PCBs, placental transfer of MeHg is more consequential than lactational transfer (Wren *et al.*, 1987a). Suckling animals have a limited ability to metabolize MeHg, leaving maturing brain cells vulnerable to toxic effects. Dietary MeHg may complex with selenium in fish, decreasing the bioavailability of MeHg. Vitamin

E also decreases MeHg toxicity. If atrazine contaminates a diet that also contains MeHg, an earlier onset of neurotoxicity is observed (ATSDR, 1994). In the wild, a food shortage may increase the toxicity of contaminants ingested (Wren *et al.*, 1987a).

Olson and Boush (1975) found that mercury present in Pacific blue marlin (*Makaira ampla*) was more neurotoxic to prenatally-exposed rats than methylmercury hydroxide fed at the same concentration (2 ppm). In this study, if fish contaminated with MeHg, rather than methylmercuric chloride, had been used in Diet C, neurotoxic symptoms might have developed in the kits.

In this study, it appeared that a mink that bit or scratched in the maze did so because it wanted to get access to the cage and the food. It showed no flexibility of changing the object or direction of its intent, venting its irritation and excitement by trying to enter where it could not. Sobotka *et al.* (1974) discussed that compressing brain development into a shortened temporal interval may limit the behavioral flexibility of the mature animal. In this study, the MeHg-treated litters started to open their eyes about a day and a half earlier than control litters. However, the kits in all treatments displayed frustration behavior, especially by scratching. This may indicate that the mink is an easily agitated animal or that biting and scratching cannot be assumed to be signs of frustration in the mink.

Stereotypic Behavior Test

Stereotypic activity in mink has been described by Bildsøe *et al.* (1990a, b; 1991), Hansen (1993), and Mason (1993b). In this study, the individual mink displaying this behavior was categorized as having "vertical" stereotypy: up and down movement of the

anterior body with the posterior part still. Other stereotypies include scratching or biting intensively at the cage wire; horizontal, or side to side movement of the anterior body with the posterior part still; mixed (vertical and horizontal); nipple, a circular movement with the head around or near the drinking nipple or cup; pendling, end-to-end of cage pacing; bottom, like pendling but with simultaneous nose-circling directed toward the cage floor; horizontal circling; vertical circling, or running from floor to wall to ceiling to wall; and jumping, usually in and out of a raised nestbox (Bildsøe *et al.*, 1990a, b; 1991; Hansen, 1993). According to Bildsøe *et al.* (1990a), females display more stereotypic behavior than males, and younger animals more than older ones. Mason (1993b) saw stereotypies exhibited by mink as young as 10 weeks (70 days) of age. In this study, the animal was 79 days old. Kits from larger litters tend to develop this behavior, possibly due to crowding and frustration (Hansen, 1993). Bildsøe *et al.* (1991) found that mink with high stereotypy levels had lower baseline but higher response cortisol levels than normal-behaving mink, suggesting that stereotyping mink are more susceptible to stress. Kits may learn stereotypies from their dam; however, neighbors have little impact on an individual's level of stereotypy (Hansen, 1993). Ambient temperatures inversely affect activity levels in general (Bildsøe *et al.*, 1990b). Why only one kit on this study displayed stereotypic behavior perhaps can be addressed by the assumption that some individuals find the environment more eliciting than others (Mason, 1993a).

Bowman *et al.* (1989) found that although rhesus monkey infants treated *in utero* and lactationally with up to 25 ppt 2,3,7,8-TCDD were more passive as infants than controls, the exposed offspring were more dominant or aggressive than controls when tested for

peer-group social behavior, which was regarded as a maladaptive sign. Mink are housed individually and therefore were not tested for social behavior in this study. None of the mink exhibited excessive self-directed behavior such as clipping or hair chewing.

SUMMARY

Environmentally altered PCBs, 0.5 ppm as Great Lakes carp, and MeHg, 0.5 ppm as methylmercuric chloride, did not statistically alter the neurobehavioral development of mink kits exposed *in utero* and via lactation. The data gathered in this study suggest, however, that the neurological development of kits exposed to PCBs was delayed and the development of those exposed to MeHg was accelerated. Specifically, it was possible that the PCB litters were born prematurely, since gestation was about one day shorter than controls. Also, PCB kits were delayed developing exploratory behavior, and the increased latency of the males in the T-maze suggests a feminization effect. Conversely, the MeHg males had a shorter latency than controls in the T-maze. Also, the MeHg kits were heavier at weaning, opened their eyes about a day and a half sooner than controls, and were more ready to explore in the open field.

Testing for righting ability and response to a tail pinch should provide valid information on reflex behavior. Similarly, biological development can be monitored by knowing age at eye opening and testing forelimb grip strength. The open-field test may not be valid when examining emotionality but may be a useful tool when analyzing exploratory behavior. In this study, gait measurement did not provide insight to motor ability. The mink kits in this study either learned well and quickly displayed the ability to reverse a learned response or they did not learn the T-maze task at all. Testing with a T-maze should produce valid results, according to the data in this study. Stereotypic behavior is only just beginning to manifest itself at the age at which the kits in this study were observed. Observing for stereotypies would be better done on older kits.

FUTURE STUDIES

Future studies which may involve testing developmental neurotoxicants in mink should consider increasing the number of dams per treatment group, in order to compensate for those that may not breed, unexpected mortality, or invalid test results. Researchers testing suspected developmental neurotoxicants should strive to use a dose that is below the LOAEL so that effects seen can be attributed to the neurotoxicity of the tested compound and not to overt health effects.

The righting ability test should be conducted at birth and three weeks of age. The kits must be conscious for this test, and too frequent handling may increase mortality due to anxiety in the dam.

The tail-pinch response test would be improved if a consistent pinch can be delivered. Perhaps a mechanical pinch, at a set pressure, would prove suitable. Also, categorizing responses may yield more information beyond simply counting them. If feasible, an electroencephalogram (EEG) could provide detailed neuroelectrical response data.

When examining the kits for eye opening, the entire litter should be checked until all kits have their eyes open. Another biological developmental parameter to monitor might be tooth eruption.

The forelimb grip strength test can be improved by quantifying the grip with a strain gauge. The mink kits tended to lean over the rod while grasping it, counterbalancing gravity. By pulling them backward by the tail while they are gripping a wire attached to a strain gauge, a numerical value can be obtained and analyzed.

In the open-field test, rather than have the floor be a grid, concentric circles would

give a clearer picture of where the kit moved in relation to the perimeter or the center of the ring. A longer test time, perhaps five minutes, might provide more information about levels of activity. Ideally, the entire test could be videotaped, reducing possible distraction by the viewer.

The gait measurement test might be improved by videotaping as well. The kit can be placed on a clear surface and the activity recorded from underneath. This would eliminate the need for painting the kit's feet, which can be stressful to the kit during handling.

In order for the T-maze test data to have strength, more kits must be used if only about 50 percent are "trainable." In this study, male kits proved to be more easily trained. Future studies might focus on male mink.

Observing for stereotypic behavior when testing suspected developmental neurotoxicants may not provide useful data as stereotypies are not uncommon in untreated mink. On the other hand, observing sexual behavior in adult mink exposed pre- or postnatally to neurotoxic compounds might disclose information relevant to survivability of wild populations. In rats exposed neonatally to Aroclor 1254 (100 μmol/kg via intraperitoneal injection), changes were observed in the activities of steroid-metabolizing enzymes (Haake-McMillan and Safe, 1991), suggesting a change in the concentration of steroids which could effect a change in sexual behavior. In mice exposed neonatally to dichlorodiphenyl trichloroethane (DDT) or PCB, the frequency of implanted ova decreased when both the male and the female of a mating pair had been nursed by DDT- or PCB-treated (three weekly subcutaneous injections of 50 mg/kg) mothers (Kihlström *et al.*, 1975). One possible explanation could be that, even at low doses, DDT and PCB would disturb the

normal sexual development by increasing the catabolism of steroids during the critical period of being suckled with milk containing DDT or PCB.

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