REPRODUCTIVE PERFORMANCE OF MICE FED WITH GREAT-LAKES CARP AND FISH FARM RAISED CARP

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ABSTRACT

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Three successive studies were performed to determine the reproductive toxicity of Great Lakes fish on mice. Mice were randomly divided into three main treatment groups: lab Chow (C), farm raised carp (A), and Great Lakes carp (G). The treatment diets were provided immediately after the dams (F-0) gave birth through lactation. Both F-1 and F-2 were fed the treatment diets from weaning to termination. Reproductive performance of both generations were examined *in vivo* and *in vitro*. Increased liver weight and decreased hepatic vitamin A and E were observed in both fish-eating groups. The highest liver weight/body weight ratio was found in group G. Group A consistently had the lowest body weight in F-1 and F-2 males. There were no differences in fecundity, lactation index, litter size, thymus and testis weights, and caudal epididymal sperm concentration, motility, and *in vitro* fertilization ability in both F-1 and F-2 males. No difference in body, liver, thymus weight, and *in vitro* fertilization in F-1 and F-2 females was observed. Based on the parameters observed in this study, Great Lakes carp did not alter the reproductive performance in young mature mice for two generations.

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INTRODUCTION

The Great Lakes represent one of the world's most significant inland freshwater systems and one of sizeable economic and ecological importance to both the United States and Canada. Unfortunately, increased commercial production and widespread use of synthetic compounds since the 1940s have contaminated this ecosystem. The widespread contamination of the Great Lakes basin has led to concern about the overall effects on the health of human and animal populations associated with this fresh water environment (Government of Canada, 1991).

Eleven persistent toxic chemicals have been identified as critical Great Lakes contaminants by the International Joint Commission (1987). They are polychlorinated biphenyls (PCBs), chlorinated pesticides (including DDT and its metabolites), dieldrin, toxaphene, tetrachlorodibenzo-*p*-dioxin (TCDD), tetrachlorodibenzo-furan (TCDF), mirex, hexachlorobenzene (HCB), mercury, alkylated lead, and benzo[a]pyrene (B[a]P). The physical and chemical properties (e.g., low volatility, chemical stability, lipid solubility, slow rate of biotransformation and degradation) of these synthetic chemicals and heavy metals allow them to gain entry into

organisms. In biological systems some persistent metabolites bioaccumulate. The biomagnification of chemicals such as PCBs, dioxins, and organochlorine pesticides from the lowest trophic levels (phytoplankton) to top predators (fish-eating animals and humans) has been observed in the Great Lakes food web.

Several recent studies have shown that humans and fish-eating animals that consume significant amounts of Great Lakes fish accumulate many contaminants found in the Great Lakes. These contaminants, such as PCBs and TCDD, are known to be detrimental to the reproductive system (Poland and Knutson, 1982). Some fish predators of Great Lakes fish like mink, bald eagles, herring gulls, lake trout, and lake turtles have experienced adverse effects from the Great Lakes contaminants on reproduction and consequent declines in populations (Government of Canada, 1991).

Reproduction is a complex, stepwise, cyclic process involving gametogenesis, gamete interaction, implantation, embryonic development, and sexual maturation of the offspring. Reproductive toxins may act directly, by virtue of either structural similarity to endogenous compounds such as hormone or chemical reactivity with biological component like alkylating agent and chelator (Welch *et al.*, 1971; Allen *et al.*, 1979; Anderson *et al.*, 1980). Other reproductive toxins may act via metabolic processing within the organism before exerting toxic effects. The metabolite formed may then exert

its toxic effect through the mechanisms of reproductive toxicity described above. Other indirect-acting reproductive toxins may produce alterations in physiological control mechanisms of the organism, such as enzyme induction or inhibition.

Reproductive toxins may exert adverse effects through more than one mechanism. For example, PCBs may act indirectly by induction of microsomal monooxygenases or UDP-glucuronyl transferase (Poland and Knutson, 1982). PCBs may also act directly by virtue of steroid hormone agonist properties (Bitman and Cecil, 1970).

The study is designed with intention to answer the question, "does consumption of chlorinated hydrocarbon and mercury contaminated Great Lakes fish pose a reproductive hazard to mammals?" Using mice as the model, the effects of Great Lakes carp-containing diets on reproduction were examined.

LITERATURE REVIEW

I. Polyhalogenated Aromatic Hydrocarbons (PAHs)

Polychlorinated biphenyls (PCBs), dibenzo-*p*-dioxins (PCDDs), dibenzofurans (PCDFs), azo(xy)benzenes, and naphthalenes collectively termed PAHs, have received increased attention in the scientific literature and popular press over the past 25 years as toxic environmental pollutants. This group of chemicals is usually considered together, because (1) their chemical structures are similar, i.e. they are approximate isostereomers; (2) they produce similar characteristics of toxic responses, although they vary greatly in potency; and (3) they are believed to act through a common mechanism (WHO, 1989c).

A. Polychlorinated Biphenyls (PCBs)

Introduction

The synthesis of PCBs was first described in 1881 by Schmidt and Schultz (Peakall and Lincer, 1970). The industrial potential applications of PCBs were not well realized until about 1930. PCB mixtures quickly gained wide acceptance in industrial products where nonflammability and heat-resistant properties were desired. Specific industrial applications of PCBs includes hydraulics, lubricants, transformers, capacitors, plasticizer applications, petroleum additives, and constituents of heat transfer systems. The

increased use of PCBs generated to a series of commercially available raw materials marketed under various trade names: Aroclor (Monsanto, U.S.A.), Clophen (Bayer, West Germany), Phenoclor (Caffaro, Italy), Kanechlor (Kanegafuchi, Japan), Pyralene (Prodelec, France), and Sovol (U.S.S.R.) to name a few. Domestic sales of the Aroclors in the U. S. increased from 32 million lbs. in 1957 to almost 80 million lbs. in 1970 (Nisbet and Satofin, 1972).

Unfortunately, the characteristics that make this class of compounds desirable for industrial use at the same time make them extremely resistant to spontaneous degradation in the environment. As a result, residues have been detected in human and animal tissue as well as in environmental specimens such as soil and river and lake sediments from diverse locations around the world. Concerns over PCB contamination of the environment and the associate toxic effects first became apparent when PCBs in fish and wildlife were reported by Jensen in 1966. This discovery prompted monitoring efforts in the United States. It soon became obvious that uncontrolled and widespread use of PCBs had led to their incorporation into the global environment as persistent and ubiquitous contaminants (Holmes *et al.*, 1967; Koeman *et al.*, 1969; Risebrough *et al.*, 1969; Risebrough and Waid, 1982).

Physical and Chemical Properties

The most popular blend of industrial grade PCBs in North America was Aroclor 1242 (Waid, 1990). Aroclors are

designated by four-digit numbers; the first two digits (12) following the trade name specify that the compound is a chlorinated biphenyl mixture; the last two digits (42) give the approximate percent of chlorine by weight in the compound. Aroclor 1016 which contained about 41 percent chlorine was an exception to this nomenclature system.

The PCBs, systematically called 1,1'-biphenyl, chloro-derivatives, consist of a group of 209 congeners ranging from monochloro- to decachloro-biphenyl. PCBs are colorless crystals when isolated in pure form by recrystallization. The solubility of PCBs in water is extremely low, especially for higher chlorinated congeners. However, all PCBs are soluble in organic solvents, oils, and fats.

Chlorinated biphenyls are quite stable to chemical alteration. From an environmental point of view, air oxidation, aqueous hydrolysis, and photochemical reactions in sunlight are potential reactions to be investigated. Existing studies showed that PCBs are fairly resistant to oxidation and hydrolysis under conditions of light, high temperatures, and enzyme degradation (Trotter, 1975).

Sources of Exposure

PCBs were included into the environment by many routes. Sources of PCBs included industrial leaks and accidents, and contamination from food-packaging materials containing PCBs.

PCBs became the most abundant of the chlorinated aromatic pollutants in the ecosystem (Risebrough *et al.*, 1968). Biota

potentially acquired PCBs from three sectors of the environment: atmosphere, water, and food (Biros *et al.*, 1970; Risebrough and Brodine, 1970).

Duke *et al.* (1970) found the source of mollusk and fish PCB (Aroclor 1254) contamination at a Florida industrial plant that leaked PCBs into the river. In 1970, Holden also reported PCB-contaminated shellfish and marine life along Scotland's Atlantic coast. He found that the contamination occurred when sludge from nearby sewage treatment plants was dumped in a deep-water estuary. Several recent surveys indicated that PCB residues occur in human populations from both industrialized and developing countries (Jensen, 1987).

Contamination Level

Due to the physical and chemical properties of PCBs, once they enter organism, bioaccumulation occurs in the food chain. The biomagnification of PCBs from the lowest trophic level to the top predators has been observed in the Great Lakes food web (Government of Canada, 1991). Since aquatic animals excrete organochlorine chemicals very slowly or not at all, these contaminants build up to higher concentrations at each step in the food web. By 1972, significant concentrations of PCBs had been discovered in animal feeds and many foods including milk, poultry, dairy products, eggs, and freshwater fish (Gold, 1983). The most contaminated food was freshwater fish. Concentrations of PCBs were determined in samples taken at different times and in different Great Lakes regions in

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fish and herring gull eggs, the results are summarized in Table 1. In lake trout, total PCB concentrations are the highest in Lake Michigan and lowest in Lake Superior. Biomagnification of PCB concentrations also can be observed, from low to high, in spottail shiners, lake trout, and herring gulls eggs in the Great Lakes.

Table 1. Mean concentrations[a] of PCBs in fish and herring gull eggs from Great Lakes.

	Lake				
Species	Ontario	Michigan	Eric	Huron	Superior
Spottail Shiners	0.028[b] (1979-1983)	NA[c]	0.15[b] (1982-1983)	< 0.01[b] (1979-1981)	< 0.01[b] (1983)
Lake Trout[d]	2.6 (1988)[e]	4.5 (1984)	NA	0.6 (1987)	0.2 (1986)
Herring Gull eggs[f]	15 (1989)	9.7 (1989)	19 (1989)	13 (1989)	7 (1989)

[a]All values are in parts per million (ppm) on a wet-weight basis.
[b]Government of Canada, (1991).
[c]Data are not available.
[d]Environment Canada and Department of Fisheries and Oceans, (1982).
[e]The numbers in parentheses refer to when the sample collected.
[f]Source: Canadian Wildlife Service, (1989).

Several researchers examined correlations between PCB body burdens in women who eat fish and different geographic locations surrounding the Great Lakes. Monitoring programs for PCBs in humans have mainly been restricted to samples of adipose tissue, breast milk and blood. Until now, human milk was investigated most frequently (Jensen, 1983; Jensen and Slorach, 1989). The differences in sampling and analysis

protocols made it extremely difficult to compare data among studies. Chemical residues measured in these studies may not truly represent body burdens in women and consequently, the degree of exposure to infants. In addition, PCB concentrations in milk samples taken from women in the same region consuming similar amounts of fish varied enormously. For example, the mean PCB concentration in milk samples from women in Toronto consuming similar amounts of fish was 25 ppb with a standard deviation of 23 ppb (Frank *et al.*, 1988). This variation may be due to the different sampling time relative to the stage of lactation, the number of previous breast-fed infants, place of residence and life history of exposure to PCBs.

Absorption

The uptake of PCBs could occur by three mechanisms (Shaw and Connell, 1990):

1. absorption of PCBs in the atmosphere/water through lungs in mammals or birds and gills of fish.
2. absorption of PCBs in the atmosphere through the epidermis.
3. consumption of food containing PCBs and passage through the gastrointestinal tract.

The atmospheric route through the lungs and epidermis is of little significance due to the very low concentrations of PCBs in the atmosphere. A primary route of uptake by fish was absorption by the gills, since the gills represent a active membrane surface for water exchange (Addison, 1976; Nadeau and

Davis, 1976; Phillips, 1980). Once absorbed by the gills, PCBs are then partitioned into the blood and then to other organs.

The major route of PCBs acquisition by animals is through contaminated food. Gastrointestinal absorption of commercial PCB mixtures and individual congeners has been investigated extensively in laboratory animal studies (Albro and Fishbein, 1972; Allen *et al.*, 1974a,b; Matthews and Anderson, 1975; Gage and Holm, 1976; Tanabe *et al.*, 1981). A commercial PCB mixture containing Kanechlor-300, 400, 500 and 600 in corn oil was administrated orally to immature male Wistar rats for 5 days (Tanabe *et al.*, 1981). More than 84 percent of the total dose was absorbed from the gastrointestinal tract with less than 16 percent of the dose excreted in the feces.

The major structural determinant that governs absorption efficiencies is the degree of chlorination. Quantitative studies of PCB absorption indicate that most, if not all, PCBs containing six or fewer chlorine atoms are efficiently absorbed by intestine in rats (Albro and Fishbein, 1972; Van Miller *et al.*, 1975). Dermal studies with PCB congeners or mixtures demonstrate that these compounds are readily absorbed and elicit toxic and biologic effects at dermal and distal sites (Nishizumi, 1978; Puhvel *et al.*, 1982; Wester *et al.*, 1983).

Distribution

For more than two decades it has been known that PCBs accumulate in human tissue. In the body, PCBs are associated with lipids; the highest concentrations are found in fat-rich tissues such as adipose tissue and milk (Jensen, 1989). However, steady state concentrations in blood, muscle, adipose tissue, and human milk are on the similar calculation basis (Brown and Lawton, 1984; Birnbaum, 1985). The initial PCB distribution in serum is dependent on blood-flow rates, blood volumes, PCB-serum absorption affinities, tissue/blood partition ratios, perfusion rates and tissue volumes (Matthews and Dedrick, 1984).

PCB transport from the application site to the distal sites occurs via several processes. The absorption of PCBs is consistent with passive absorption into the lipophilic cell membranes followed by transport to all tissues via the blood (Maliwal and Guthrie, 1982). Liver and muscle are the primary early depots because the liver is highly perfused and has moderate affinity for these compounds. Muscle makes up the largest tissue volume. However, since PCBs are highly lipid-soluble compounds, they have a higher affinity for lipid-rich tissues other than the liver and muscle (Hansen and Welborn, 1977). Therefore, these compounds are eventually concentrated in adipose tissue and the skin (Matthews and Tuey, 1980).

It was also apparent that tissue persistence of individual PCB congeners are dependant on their structure and

the metabolism rate. In addition to the degree of chlorination of individual PCBs, orientation and chlorine substitution patterns are also important structural features that determine tissue persistence (Safe, 1989).

Metabolism and Elimination

Generally, the tissue mixtures from humans and animals containing PCBs from environmental exposure have GC patterns resembling those of PCB mixtures with more than 50 percent chlorination (Matthews *et al.*, 1978). This is in marked contrast to the major manufactured products containing 42 percent or less chlorine. While one cannot rule out the possibility that differential uses favor introduction of more highly chlorinated materials in the environment, the observation has led to the general belief that less-chlorinated components are more readily metabolized than highly chlorinated congeners.

Environmental observations have been confirmed in many experimental feeding studies using a number of mammalian and avian species. The absence or diminished concentration of the early eluting peaks were reported in rats (Curley *et al.*, 1971), rabbits (Grant *et al.*, 1971), cows (Fries *et al.*, 1973a), quail (Bailey and Bunyan, 1972), and hens (Fries *et al.*, 1973b). The peaks absent in tissues and products generally are the early eluting peaks that correspond to PCBs with lower degrees of chlorination. In a rat study, Matthews and Anderson (1975) injected intravenously ^{14}C-labeled PCBs

(4-chloro-, 4,4'-dichloro-, 2,3,5,2',5'-pentachloro- and 2,4,5,2',4',5'-hexachlorobiphenyl) at a dose of 0.6 mg PCBs/kg of body weight to determine the total radioactivity in major organs and tissues from 15 minutes to 42 days later. An increase in PCB retention with increasing ring chlorination was reported in this study. This observation was consistent with the belief that the metabolism rate of PCBs decreases with increasing chlorination. Studies of single PCB homologs at various degrees of chlorination have shown that those with five or fewer chlorine atoms are more readily metabolized and excreted than those with higher chlorination (Hutzinger *et al.*, 1972; Berlin *et al.*, 1975).

The effects of chlorine substitution patterns in sites of oxidation have not been studied systematically. Examination of the results in the literature, however, suggest the following: (1) hydroxylation is favored at the *para* position in the least-chlorinated phenyl ring unless this site is sterically hindered; (2) in the lower chlorinated biphenyls, the *para* position of both biphenyl rings and carbon atoms, which are *para* to the chloro substitute are all readily hydroxylated; (3) the availability of two vicinal unsubstituted carbon atoms, particularly C-5 and C-4 in the biphenyl nucleus, also facilitates oxidative metabolism of the PCB substrate, but is not a necessary requirement for metabolism; (4) as the degree of chlorination increases on both phenyl rings, the rate of metabolism decreases; and (5)

the metabolism of specific PCB isomers by different species may result in considerable variations in metabolite distribution (Safe, 1989).

The metabolism of individual PCBs by induced and non-induced rodent and human liver microsomes (Ghiasuddin *et al.*, 1976; Shimada, 1976; Kennedy *et al.*, 1980; Kaminsky *et al.*, 1981) and rat hepatocytes (Vickers *et al.*, 1986) was reported. In the studies by Kaminsky *et al.* (1981), it was evident that the metabolism of several dichlorobiphenyls was dependent on the substrate structure and the type of cytochrome P-450 isozymes. For example, the major phenobarbital-induced cytochrome P-450 isozyme preferentially metabolizes di-*ortho*-chloro-substituted biphenyls, whereas the ß-naphthoflavone-induced cytochrome P-450 isozymes primarily metabolize dichlorobiphenyls, which do not contain *ortho* substitutes. Both cytochrome P-450 isozymes catalyze the oxidation of mono-*ortho*-chloro-substituted dichlorobiphenyls.

Due to their high lipid solubility, PCBs are most concentrated in substances with a high lipid content. They have been detected in oils on human and animal hairs (Matthews *et al.*, 1976). However, the major carriers for PCB elimination are substances with the greatest volume and/or lipid content. Lactation is the most important elimination route for such chemicals (Brilliant *et al.*, 1978; Yakushiji *et al.*, 1979; Nau *et al.*, 1986). Through the transfer of residues accumulated over a lifetime from mother to offspring,

PCBs may be retained in animal populations for several generations. Vodicnik and Lech (1982) found lactating mice transferred most of the body burden to their nursing young during a period of 20 days, whereas the body burden of the non-lactating animals remained essentially constant.

Eggs, particularly egg yolks, represent another carrier of newly synthesized lipids that may serve as a route for PCB elimination. PCBs have been detected in the eggs of fish (Johnson and Morris, 1974) and wild birds (Koivusaari *et al.*, 1972). Trout exposed to PCBs experimentally had a more rapid elimination rate in both sexes during spawning; this appeared to be primarily due to the voiding of PCB-containing eggs and sperm (Harding and Addison, 1990).

Toxicity

a. Clinical Cases:

The first incident in which PCBs were recognized as the causative agent in intoxicating the general public happened in Japan in 1968 (Higuchi, 1976). A PCB mixture used in a rice oil plant's cooling system leaked into rice oil subsequently consumed by over 1,600 persons. Another accidental exposure of humans to PCBs occurred in Taiwan in 1979 (Chen *et al.*, 1980). The PCB concentrations in toxic rice oils causing PCB poisoning were about 830 to 1,030 ppm in Japan (Nagayama *et al.*, 1975) and 53 to 99 ppm in Taiwan (Chen *et al.*, 1981). The blood-PCB concentrations of PCB-intoxicated patients in Japan were about 5.9 ppb (Koda and Masuda, 1975), much lower

than those of Chinese patients. The mean blood PCB value of Chinese patients was 49 ppb (Chen *et al.*, 1980). This large difference is presumably due to the difference in time lags between PCB intoxication and blood-PCB analysis. For Japanese patients, the blood PCB analysis was done about 5 years after toxic rice oil ingestion; for Chinese patients the blood PCB measurement was made 9 months to 1 year after intoxication. The patients reported in these two cases developed a spectrum of adverse symptoms, such as chloracne, gum and nailbed discoloration, joint swelling, waxy secretions of the glands in the eyelids, lethargy and joint pain (Urabe *et al.*, 1979; Chen *et al.*, 1981).

An ongoing study involving the offspring of women who consumed Lake Michigan fish demonstrates the occurrence of several effects. The children were born to women who consumed at least 11.8 kg of Lake Michigan fish, equivalent to 2 to 4 fish meals monthly, over at least 6 years. Maternal serum PCB concentrations averaged 5.5 ± 3.7 ng/mL; umbilical cord serum PCB levels averaged 2.5 ± 1.9 ng/mL (Fein *et al.*, 1984). In this study, 242 women showed significantly decreased in infant birth weight (160-190 g lighter than controls), gestational length (average 4.9 days less than controls) and head circumference (average 0.6 cm smaller than controls) (Fein *et al.*, 1984). Consumption of contaminated fish also contributed to motor immaturity, poorer lability of states, a greater amount of startle and more abnormally weak reflexes. The most

highly exposed infants were frequently described as "worrisome" (Jacobson *et al.*, 1984). The infants were retested at 7 months of age and a significant decrease in visual recognition of novel visual stimuli was associated with increased levels of PCB cord serum (0.2 to 7.9 ng/ml). This decrement did not correlate with fish consumption during pregnancy or lactation (Jacobson *et al.*, 1985). Further studies of these children have demonstrated that PCBs are still detectable in a significant proportion (4.18 ± 3.29 ng/ml) of the serum samples from the children at 4 years of ages. Mothers' milk was the primary source of exposures (Jacobson *et al.*, 1989). However, prenatal exposure predicted poor short-term memory on both verbal and quantitative tests in a dose-dependent manner (Jacobson *et al.*, 1990). Although much larger quantities of contaminants are transferred postnatally via lactation, prenatal exposure seems more important in terms of developmental effects.

b. Effects on Enzyme Activity:

One of the major biochemical effects of PCBs is the induction of microsomal enzymes in the liver. Risebrough *et al.* (1968) suggested that PCBs have the capability to induce microsomal enzyme activities. Street *et al.* (1969) demonstrated the induction of liver enzymes in rats by PCBs. Since then, many articles have been published on this subject (Litterst and Van Loon, 1972; Chen *et al.*, 1973; Allen *et al.*, 1974a; Turner and Green, 1974). The enzyme systems studied

have included mainly hydroxylases, *N*- and *O*-demethylases and nitroreductases and, to a lesser extent nonspecific carboxylesterase, bromosulfophthalein-glutathione conjugating enzyme, p-nitrophenol UDP-glucuronyl transferase and EPN-detoxification systems.

Two types of enzyme inducers are reported in the literature. One group, to which phenobarbital belongs, increased cytochrome P-450 content, and increased benzopyrene hydroxylase and ethylmorphine demethylase activities in the liver (Alvares *et al.*, 1970; Bresnick, 1978; Cinti, 1978). The second group stimulated the formation of cytochrome P-448 and an increase in hydroxylation but not demethylation (Conney, 1967; Lu *et al.*, 1980). Alvaers *et al.* (1973) reported that rats treated with PCBs produced an increase in cytochrome P-448, hydroxylase, and demethylase. Therefore, PCBs display induction behavior typical of both groups.

c. Carcinogenicity:

Most chemicals are not carcinogenic unless they undergo metabolic activation to become highly reactive electrophilic intermediates with the capacity to bind covalently to cellular constituents such as RNA, DNA, and proteins (Heidelberger, 1975). The formation of an arene oxide intermediate during the metabolism of PCBs was proven previously (Safe *et al.*, 1975; Sundström and Hutzinger, 1976). Arene oxides are electrophilic in nature. They may be toxic by forming adducts with macromolecules in cells and interfere with cellular

functions. They may also detoxify other toxicants by facilitating the formation of glutathione conjugates and other phase II conjugates. The *in vivo* binding of 2,2',4,4',5,5'- and 2,2',3,3',5,5'-hexachlorobiphenyl to hepatic proteins, RNA and DNA in mice has been demonstrated (Morales and Matthews, 1979). The greatest binding was observed in RNA, followed by protein and DNA, respectively. *In vitro* studies using mammalian cells in culture showed DNA damage mediated by some PCB congeners and their metabolites (Seymour *et al.*, 1976; Shimada and Sato, 1978). The DNA was isolated and examined for strand breaks by centrifugation techniques. All of the compounds tested (2,5,2'5'-tetrachloro biphenyl, 4'-chloro-4-biphenylol, 4'-chloro-3-methoxy-4-biphenylol, 4'-chloro-4-methoxy-4-biphenylol, 4'-chloro-3,4-biphenyldiol) induced single-strand breaks in L-929 cell DNA.

d. Immune Alterations:

Polyhalogenated aromatic hydrocarbons (PCBs, PBBs, PCDDs, PCDFs, etc.) have immunotoxic properties (Vos and Luster, 1989). The first suggestion that PCBs might affect the immune system stemmed from an observation on weight and histological changes of lymphoid organs, including the thymus, spleen and lymph nodes (Vos *et al.*, 1980). More direct evidence that PCBs may alter immune responses was obtained using functional (antibody titration) tests (Vos and de Roij, 1972). Exposure of guinea pigs to Clophen A60 for 4-8 weeks reduced serum antibody titers to tetanus toxoid. Loose *et al.* (1977)

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reported that exposure of mice to Aroclor 1242 for 6 weeks suppressed both primary and secondary antibody responses to sheep red blood cells and abolished memory cell functions. In addition, they reported that PCB exposure lowered circulating immunoglobulin levels. Acute PCB exposure also reduced antibody synthesis. In general, the immune alterations in exposed animals resulting from PCB poisoning included depression of serum immunoglobulin levels and delayed-type hypersensitivity reactions, as well as enhanced *in vitro* proliferation responses of T-lymphocytes.

e. Reproductive Toxicity:

PCB effects on reproduction were first shown by Gilbert (1969). Later, a severe decrease in the reproduction and survival rates of mink were observed in commercial ranches where a diet containing PCB-contaminated fish was used (Aulerich *et al.*, 1971). Then, Ringer *et al.* (1972) fed 10 ppm Aroclor 1254 to mink, the same concentrations as those contained in Lake Michigan Coho salmon, and confirmed the hypothesis that PCBs were causative agents of reproductive failure in mink. When groups of 12 female and 4 male mink were fed diets containing 0, 1, 5, or 15 ppm Aroclor 1254 for 4 months and were mated, a dose-related impaired reproduction (reduced number of females whelped and reduced kit/female ratio) occurred at 5 ppm, with total inhibition of reproduction at 15 ppm (Aulerich and Ringer, 1977).

Adverse effects of PCBs on the estrous cycle, fertility,

growth and mortality were reported in rats (Brezner *et al.*, 1984). Female Wistar rats were orally administered 30 mg Aroclor 1254/kg body weight for one month. The estrous cycle was prolonged in 67 percent of the rats. Sexual receptivity and litter size decreased. Vaginal bleeding was frequent during gestation and parturition was delayed. Offspring, whether exposed prenatally and/or postnatally, demonstrated lower body weight gain than controls and a higher mortality rate.

In contrast to rats, Swiss-Webster mice fed 10, 100 or 250 ppm Aroclor 1254 alone or in combination with lead for 12 weeks prior to breeding and during gestation and lactation showed no adverse effects (Talcott and Koller, 1983). No effects were observed on reproduction or the pups' immune response to antigen challenges.

A single dose of Aroclor 1254 (125-1000 ppm) administered to male mice had no effect on sperm head abnormalities observed 5 weeks later (Tophan, 1980). Studying the effects of PCBs on sperm production, Sanders *et al.* (1977) reported that Aroclor 1254 in the diet of adult albino mice at a concentration of 200 ppm for 15 days significantly reduced the number of spermatozoa in testes, but did not affect gonadal weights. Nor did they find any effects at 50 ppm after a 15-day treatment period. A similar effect on reduction of spermatozoa from 400 ppm Aroclor 1254 in the diet was observed in adult, white-footed male mice (Sanders and Kirkpatrick,

1975). No decreases in the 100 and 200 ppm treatment groups was observed.

In a study of the effects of PCBs on fertility, Orberg (1977) administered daily oral doses of 50 μg of either trichlorobiphenyl or hexachlorobiphenyl to mice from days 5 to 19 of gestation or from birth through day 12 of lactation. Male pups were subsequently mated with nontreated females. The numbers of pups sired were not affected. Kihlstrom *et al.* (1975) injected postpartum NMRI strain mice with 50 mg Clophen A-60/kg body weight. Treatment began on the day of delivery and then weekly for 3 successive weeks during lactation. After reaching sexual maturity, the male offspring were mated with nontreated females. The frequency of implantation and the resorption rate were not different from controls. When treated females were mated to control males, there was no effect on implantation, either. Interestingly, when both treated males and females were mated, the number of implantations decreased significantly.

Despite numerous studies concerning the adverse effects of PCBs on mammalian reproduction, mechanisms and/or sites of action remained unknown. Reproductive failure may be caused directly via a toxic PCB action on reproductive organs and cells or indirectly via a lesion in the neuro-gonadal axis (Fuller and Hobson, 1986). Demonstrations of PCB accumulation in gonads in conjunction with reports of altered steroid hormone regulations in various mammalian models suggest

effects of PCBs on endocrine function (Fuller and Hobson, 1986).

An intriguing effect of PCBs on steroidogenesis was reported by Fuller *et al.* (1980). Luteinized ovaries were induced by PMSG-hCG priming of immature albino rats. Following formation of corpora lutea, 20 mg Clophen A-30/kg body weight/day were administered intraperitoneally for 2 consecutive days. The ovaries were removed, sliced, and incubated for 2 hours in the presence of luteinizing hormone (LH). LH has the ability to increase ovarian progesterone synthesis (Hadley, 1988). Addition of LH to vials containing PCB-exposed luteal tissue increased progesterone synthesis almost 100% above basal levels; whereas, there was only a 31% increase progesterone from luteal tissue in the presence of LH alone. This suggested a synergistic relationship between PCB and LH.

Low chlorinated PCBs possess weak estrogenic activity. Bitman and Cecil (1970) found that both DDT and PCBs (Aroclors 1221, 1232, 1242, and 1248) had estrogenic activity which was reflected by an increase in glycogen content of the uterus of immature rats. Ecobichon and MacKenzie (1974) tested the effects of several Aroclors and isometrically pure chlorobiphenyls (mono-, di-, tetra-, and hexa-CB) on uterine weight, water content, and glycogen concentration in immature rats. In all Aroclor-treated rats, except Aroclor 1016, the uterine weight was increased. Of the pure chlorobiphenyls,

only those chlorinated in the 2 position affected the uterus. Among those compounds tested, decreasing estrogenic activity was noted with increasing chlorination, except 2,4,6,2',4',6'-hexachlorobiphenyl, which increased uterine weight.

Recently, it was demonstrated that PCBs will bind to estrogen receptor. Korach *et al.* (1987) tested a series of PCBs for their binding activities to soluble uterine estrogen receptor protein and found that conformational restricted hydroxy PCBs were shown to be particularly effective binding ligands for estrogen receptors. It was reported that conformational restriction may improve the estrogen receptor-binding activity of the biphenyl molecule. The degree of enhancement appears to be near maximum with one *ortho*-chlorine and with slight improvement with two *ortho*-chlorines (Korach *et al.*, 1987).

Therefore, weak estrogenic effects of PCBs could affect endocrine function by competitive affinity for hormone receptors. Altered receptor availability and/or increased hormone metabolism could change trophic hormone thresholds, thus could disrupt feedback patterns between the endocrine organs and hypothalamus. This may directly or indirectly modify some phase of the reproductive process.

Summary of PCBs

Although PCB production in the United States was banned in 1977, use of PCBs in other countries has continued. PCBs are persistent in the environment and in organisms. There are

considerable differences in toxicity among congeners and, for a given congener, there are significant species differences in sensitivity. Although metabolism and excretion of PCBs are not efficient, they are considered important modes of PCB detoxification. PCB accumulation in target organs to a great extent is closely related to chemical solubility and lipophilicity. Solubility tends to decrease with increased halogenation of PCBs, the rate of metabolism and therefore elimination. Thus, highly halogenated PCBs tend to be more persistent and have greater potential for bioaccumulation.

B. Polychlorinated Dibenzo-*p*-dioxins (PCDDs)

PCDDs, the most potent compounds of PAHs, serve as the prototype of halogenated aromatic hydrocarbons. There are 75 possible chlorine-substituted dibenzo-p-dioxin isomers. These compounds are not synthesized for commercial purposes, but are formed as trace contaminants in the synthesis of several commercial products, most importantly, chlorophenols.

There is a commonality of toxic responses produced by halogenated aromatic hydrocarbons. This is difficult to appreciate in the literature because toxic effects observed after administration of a given compound vary with the dose, duration of exposure and, most importantly, with the species of animals used. Species vary greatly in their sensitivity to halogenated aromatic hydrocarbons. The oral LD_{50} (in $\mu g/kg$ body weight) of TCDD varies over a 5000-fold range in

different species: guinea pig, 1; rat (male), 22, (female), 45; mouse, 114; rabbit, 115; hamster, 5000 (Schwetz *et al.*, 1973; Vos *et al.*, 1974; Henck *et al.*, 1981). The large species variation of TCDD sensitivity is not accounted for by an appreciable difference in the rate of TCDD metabolism. The LD_{50}s in the guinea pig and hamster differed by more than 3 orders of magnitude, but the whole body half-life in them differed only 3 fold (Olson *et al.*, 1980).

After an acute lethal dose of TCDD or other dioxin congeners, tested animals (chicken, cow, guinea pig, hamster, rabbit, rat) have a latent period of a week or more prior to death in which weight loss or reduced weight gain was accompanied by a depletion of adipose tissue. By death, the loss in body weight may be as great as 50% (McConnell, 1980). Reduction in body weight and adipose tissue were partially attributed to lack of food intake (Harris *et al*, 1973).

In all species studied, TCDD and other congeners produced a loss of lymphoid tissue, especially in the thymus, and also in the spleen and lymph nodes (Poland and Knutson, 1982). The thymus may be reduced to 0 percent of the normal size, with a loss of cortical lymphocytes. In young animals (rats, mice, and guinea pigs), TCDD-produced thymic loss was accompanied by suppression of the immune response (Faith and Moore, 1977; Vos *et al.*, 1980). The lack of effects of TCDD on primary lymphocytes and thymic cell lines *in vitro* suggests that halogenated aromatic hydrocarbons do not act directly on T

cells (Vos *et al.*, 1980), but may reduce thymocytes by acting indirectly. Lethally exposed animals, however, generally do not die from infections, nor does a germ-free environment protect them from death (Greig *et al.*, 1973).

TCDD and related compounds produce hepatomegaly in all species, even at doses well below the lethal dose (McConnell, 1980). The enlarged liver is due to hyperplasia and hypertrophy of parenchymal cells, due especially to proliferation of the smooth endoplasmic reticulum (Fowler *et al.*, 1973). This morphologic change is accompanied by increased microsomal monooxygenase activity (Poland and Knutson, 1982). In the rat liver, TCDD increased DNA synthesis and the content of DNA (Dickins *et al.*, 1981).

Chronic exposure to halogenated aromatic hydrocarbons impairs reproduction (Poland and Knutson, 1982). Compared with controls, female rats fed diets containing TCDD had fewer vaginal plugs when caged with males, longer intervals between mating and parturition, and smaller litter sizes at birth (Murray *et al.*, 1979). Decreased uterine size, decreased number of corpora lutea, and aberrant ovarian stroma cells observed in TCDD- and PCB-exposed rats suggest that estrous cycles may be altered (Kociba *et al.*, 1978). Decreases in plasma progesterone concentrations were observed in both TCDD-treated monkeys (Barsotti *et al.*, 1979) and PCB-treated rats (Jonsson *et al.*, 1976).

Enzyme induction is the most studied and best understood

response produced by PAHs, and provides the basis for subsequent formulation of their mechanism of toxicity. The microsomal monooxygenase system metabolized most exogenous lipophilic chemicals to more polar and readily excretable products (Nebert *et al.*, 1981). This enzyme complex, embedded in the endoplasmic reticulum, consisted of a flavoprotein NADPH-cytochrome P-450 reductase, and a group of hemoproteins, collectively termed cytochromes P-450 (Nebert *et al.*, 1981).

Much of the research reported to date has focused on the ability of TCDD and PAHs to bind to a specific cytosolic receptor, aromatic hydrocarbon receptor (AhR), that activates a particular enzyme locus. The Ah receptor was detectable in many tissues and organs. The best understood activity of the receptor concerns its role in the induction of cytochrome P450IA1 gene -- termed xenobiotic responsive elements -- and the stimulated transcription of this gene (Hoffman *et al.*, 1991). The receptor also mediates induction of cytochrome P450IA and several other enzymes that metabolize xenobiotics. Many pathological effects of the polychlorinated aromatic compounds depend on the action of the Ah receptor, but the pathogenetic mechanism is still unknown (Poland and Knutson, 1982). These pathological effects include hyperplasia and necrosis in the liver, proliferation in the epithelial lining in the urinary tract, loss of lymphoid tissue, and hyperplasia and hyperkeratosis in interfollicular epidermis.

TCDD produced a dose-related induction of hepatic aryl

hydrocarbon hydroxylase (AHH) activity in chicken embryos (Poland and Glover, 1973). The ED_{50} was 0.3 X 10^{-9} mol/kg body weight. The most important observation from these studies was that for chlorinated dibenzo-*p*-dioxin congeners, there was a high correlation between their potency to induce AHH activity and their toxic potency (Poland and Glover, 1973; Kende *et al.*, 1974).

II. Chlorinated Pesticides

A. Hexachlorobenzene (HCB)

Hexachlorobenzene (HCB) is a white crystal with a molecular weight of 284.80 and a melting point of 231°C (Merck Index, 1989). Chlorinated hydrocarbons were used widely as a fungicide in the world during the late 1940s and 1950s. It is also an impurity in several widely used pesticides such as tetrachlorobenzene and pentachlorobenzene (Morris and Cabral, 1986). In most countries, HCB has not been produced as a fungicide since the mid-1970s to early 1980s. Today, the major source of HCB appears to be industrial wastes originating from the manufacture of many chlorinated compounds.

HCB is highly resistant to environmental degradation. Like many other chlorinated compounds, it accumulates in food chains (Ault *et al.*, 1985). Using chemical and environmental modelling, the behavior and fate of HCB in the environment

have been demonstrated. HCB has been identified in the air, water and soil, translocating from one medium to another (Eisenreich *et al.*, 1981; Rippen *et al.*, 1984). For example, HCB may move from soils or other point sources via water run-off and concentrate in reservoirs; there it may be further taken up and concentrated by various biota and aquatic species. HCB may therefore enter the food chain via both aquatic and terrestrial plants and animals (Ault *et al.*, 1985).

During 1979-1981, HCB was detected in nearly 100 percent of the human population sampled in U.S.A., prompting efforts to identify its sources (Mack and Mohadjer, 1985). Using annual market-basket analysis, HCB residues were found regularly in cooking oil, fish, poultry, dairy products, and root vegetables (Corneliussen, 1972).

Between 1955 and 1959, the consumption of seed-wheat treated with HCB led to severe illness in 4,000 persons in Turkey. The estimated intake of HCB was 50 to 2,000 mg/day for a long time during this period (Schmid, 1960). The illness was characterized by symptoms of porphyria (Cam, 1960). Hepatomegaly was also observed in most hospitalized patients; some 30 percent of the cases showed enlarged thyroid glands (Cam and Nigogosyan, 1963). Young children were particularly at risk, nursing infants developing a lesion known as "pink sore" that was associated with a 95 percent mortality rate. HCB ingestion was related to transplacental

and milk acquisition from women who had consumed contaminated grain.

In animal models, several adverse effects were observed. A dose-dependent increase in hepatic and thyroid tumors was observed in hamsters during a chronic (70-week) study (Lambrecht *et al.*, 1982). With continued administration for 3 to 12 weeks, the activities of ethoryresorufin *O*-deethylase and glutathione-*S*-transferase were induced by HCB (Wada *et al.*, 1968; Sweeney *et al.*, 1971; Debets *et al.*, 1981), with an increase in liver size and weight (Debets *et al.*, 1981). Cutaneous lesions may occur, as well as the onset of porphyria (Smith *et al*, 1979; Debets *et al.*, 1981). Gross and microscopic observations indicated marked hepatosplenomegaly, and congestion in most abdominal and thoracic organs as early as 4 weeks after beginning the exposure to HCB (den Tonkelaar and van Esch, 1974). Enlargement of the thymus, spleen, as well as lymphoid centres and nodes were also observed.

HCB has been reported to be teratogenic in some animal species. Exposure during gestation resulted in enlarged kidneys in pups on days 1, 15 and 20 after birth. In a two-generation study in rats with concentrations of 0.3-40 ppm HCB in the diet, chronic nephrosis was observed, particularly in the males (Arnold *et al*, 1986). Parathyroid adenomas were observed in males receiving the highest dose, while neoplastic nodules in the liver (Smith *et al.*, 1986) and adrenal pheochromocytomas were observed in females receiving the

highest dose (Kuiper-Goodman and Grant, 1975).

B. Aldrin and Dieldrin

Aldrin and dieldrin are organochlorine pesticides manufactured since 1950 and were used throughout the world until the early 1970s. Both compounds were used as insecticides to control soil pests and to preserve seeds (WHO, 1989a). While readily metabolized to dieldrin in plants and animals, aldrin is rarely found in food (Worthing and Walker, 1983). Dieldrin, however, is extremely persistent in the environment and is commonly found in dairy products, meat products, fish, oils, and root vegetables (Johnson and Manske, 1977; Wessels, 1978; Zabik *et al.*, 1979; Frank *et al.*, 1985). Dieldrin/aldrin has a low propensity for movement away from treated soils (Herzel, 1972; El Beit *et al.*, 1981). Dieldrin is mainly and rapidly adsorbed on soils with a high organic matter content (Powell *et al.*, 1979).

Since the early 1970s, both aldrin and dieldrin have been restricted or banned in a number of countries because of persistence in the environment (IARC, 1974). Neither aldrin nor dieldrin is currently produced in the United States (Meister, 1987).

Concentrations of dieldrin in the adipose tissue of humans were declined from the mid-1970s to the early 1980s in the Netherlands, the United Kingdom, and U.S.A. (GIFAP, 1984). Data on dieldrin in human milk were below 10 μg/kg milk (limit

of detection) in the U.S.A. during the period 1971-1979 (National Food Administration, 1982). As a result of transplacental and lactational transfer, dieldrin was detected in the blood and adipose tissue of fetuses and newborn infants (Eckenhausen *et al.*, 1981; WHO, 1989a). The concentrations were one-tenth to one-half of that in maternal tissues.

Oral LD_{50} of dieldrin in mice and rats ranges from 40 to 70 mg/kg body weight (Cholakes *et al.*, 1981). Like most other chemical substances, dieldrin has multiple targets of toxicity. The liver is a major target organ of dieldrin toxicity in rats and mice. An increased liver/body weight ratio and hypertrophy of the centrilobular hepatocytes were observed (Benitz *et al.*, 1977). The other target is the central nervous system. In humans and other vertebrates, intoxication following acute or long-term exposure to dieldrin is characterized by headache, nausea and vomiting, followed by involuntary muscle movements and epileptiform convulsions. Death may result from cerebral anoxaemia (Jager, 1970; Joy, 1976; Hayes, 1982).

In the liver of dieldrin-treated immature male rats, there was increased activity of microsomal *N*-demethylase, dimethylaminoantipyrin, aldrin epoxidase and cytochrome P-450 contents (Campbell *et al.*, 1983). In rhesus monkeys, the activity of liver microsomal monooxygenase and cytochrome P-450 was increased by dieldrin at daily feeding doses of 1.75 or 5 mg/kg diet for approximately 6 years (Wright *et al.*,

1978). In humans, no evidence of enzyme induction was observed in a group of 10 workers in a manufacturing plant with a mean exposure equivalent to a daily oral intake of 17 μg/kg body weight (maximum 24 μg/kg body weight) (Hunter and Robinson, 1967; Hunter *et al.*, 1969).

A number of long-term carcinogenicity studies of aldrin or dieldrin in different strains of mice were conducted. In all studies, benign and/or malignant liver cell tumors were found. Females are less sensitive than males (NCI, 1978). No other types of tumors were induced. Using cultured mouse FM3A cells, Morita and Umeda (1984) reported that aldrin and dieldrin were weak mutagens. However, in a study at the National Institute of Public Health of the Netherlands, no increased incidence of tumors was found in rats fed diets containing 75 ppm dieldrin for 2 years (Van Genderen, 1979). When Syrian golden hamsters were fed diets containing dieldrin at 20, 60, or 80 ppm for 10 weeks, there was no significant increase in tumor incidence (Cabral *et al.*, 1979). Therefore, from long-term feeding experiments, it seems that aldrin and dieldrin may be carcinogenic in mice, but not in rats or hamsters. There was inadequate evidence of carcinogenicity in humans, and only limited evidence for carcinogenicity in experimental animals.

In a study by Keplinger *et al.* (1970), Swiss mice were fed a diet containing 3 ppm-dieldrin for 6 generations and there were no effects on fecundity, gestation period, or

litter sizes. However, there was an increase in the mortality rate in pre-weaning pups. Increased pre-weaning mortality in the F-1 litter was also observed in the study of Eisenlord *et al.* (1967). They did not observe any effect on fertility or litter size in a three-generation feeding study in Long-Evans rats fed 0.1, 1.0, or 2.0 ppm dieldrin in the diet. The no-observed-adverse-effect-levels for reproductive toxicity of dieldrin are 2 ppm in the rat diet and 3 ppm in the mouse diet, which are equivalent to a daily intake of 0.1 and 0.4 mg/kg of body weight, respectively.

No evidence of teratogenicity of dieldrin was found in the mouse, rat, or rabbit, after ingestion up to 6 mg/kg of body weight (Dix and Wilson, 1971; Dix *et al.*, 1978; Coulston *et al.*, 1980).

C. Dichlorodiphenyltrichloroethane (DDT)

Dichlorodiphenyltrichloroethane (DDT) was one of the most widely used pesticides to control insects on agricultural crops that carry diseases (WHO, 1979). DDT is a white, tasteless, nearly odorless crystal. DDT is stable under most environmental conditions and is resistant to complete breakdown by the enzymes present in soil microorganisms and higher organisms (WHO, 1979; 1989b). High lipid solubility and low water solubility of DDT and its metabolites enable these compounds to be readily taken up by organisms and lead to retention in the fatty tissues of fish, birds, and mammals

(Edmundson *et al.*, 1972). Rates of accumulation into organisms vary with the species, duration and concentration of exposure, and environmental conditions. In general, organisms at higher trophic levels tend to contain higher concentrations of DDT-type compounds than those at lower trophic levels (Johnson *et al.*, 1971; WHO, 1989b).

DDT was introduced into North America in 1946 and was banned in the United States in 1972. The levels of DDT and its principal metabolite, DDE, in the Great Lakes basin have decreased significantly since 1978 (Government of Canada, 1991). Since the 1970s, the level of DDT in lake trout has declined followed by a leveling off in the Great Lakes. In Lake Michigan trout, DDT concentration was decreased from 7 ppm in 1979 to 2 ppm in 1984. From recent data, concentrations of DDT/DDE in the lake trout are below 2 ppm in the Great Lakes and do not pose a hazard to human health (Government of Canada, 1991).

DDT is highly toxic to fish (Mayer, 1987). Although the body burden of DDT/DDE in lake trout can be more than 7 ppm, the acute LD_{50} reported in rainbow trout exposed to a single oral dose of DDT was 0.007 ppm (Pimentel, 1971). Adipose tissue is believed to be the site of DDT/DDE storage. Smaller fish are more susceptible than larger ones of the same species. Cellular respiration may be the main target of DDT toxicity in aquatic animals since there are reports of adverse effects on ATPase. It was demonstrated that the inhibitory

activities of DDT on ATPase eventually result in impairment of fluid absorption of intestine (Koch, 1969; Cutkomp *et al.*, 1971; Desaiah *et al.*, 1975).

A single oral dose of 237 ppm DDT caused death in mice (Bathe *et al.*, 1976). The primary effects associated with DDT and DDE exposure in experimental animals, such as rats, mice, guinea pigs, and rabbits, were liver and neurological effects (EPA, 1989). Liver effects ranged from increased P-450 enzymes to necrosis and tumors, and neurological effects, including tremors and convulsions.

Reproductive effects were observed in experimental animals following exposure to DDT. Decreased testicular weight and sperm concentration were reported in male rats after oral exposure to 500 mg DDT/kg body weight/day on days 4 and 5 of age, then 200 mg DDT/kg body weight/day from days 4 to 23 (Krause *et al.*, 1975). Female mice exposed to 1.3 to 6.5 ppm DDT for 5 generations had increased abortions, stillbirths, and pup mortality. Most of the females in the 6.5 ppm group died before parturition (Shabad *et al.*, 1973). Increased uterine weight and glycogen content were reported by Clement and Okey (1972) in immature rats that ingested 50 mg DDT/kg body weight for 7 days.

DDT and its metabolites can lower the reproduction of birds by causing eggshell thinning, leading to egg breakage, and embryo death (Lincer, 1975; Heinz, 1976). Different species of birds vary greatly in sensitivity to these

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chemicals. Predatory birds are extremely sensitive and often show marked shell thinning, whereas gallinaceous birds are relatively insensitive (WHO, 1989b). As for the mechanism of the eggshell thinning phenomenon, no generally accepted explanation exists. There are reports that DDT inhibits carbonic anhydrase which is important in forming eggshell (Peakall, 1970). Others believed that DDT's estrogenic activity causes hormone imbalance, thus changing calcium partition (Matsumura, 1985).

DDT and its metabolites have been found in human blood, placental tissue, and umbilical cord blood. Saxena *et al.* (1983) reported increased DDT levels in maternal blood ranging from 7.4 to 393.8 ppb and levels in placental tissue ranging from 19.8 to 16.2 ppb in mothers who gave birth to premature infants or who spontaneously aborted fetuses. O'Leary *et al.* (1970) reported a difference in DDE blood levels between full-term and premature infants. Women who delivered early had infants with mean DDE blood levels of 19 to 22.1 ppb, while women who delivered at full-term had infants with mean DDE blood levels of 4.9 to 6.1 ppb. High DDE level in breast milk was found to be associated with hyporeflexia in newborn infants (O'Leary *et al.*, 1972). Further investigation is needed to substantiate this finding and to determine if DDE is the causative agent.

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III. Mercury

Introduction

Sediments in areas downstream from municipal, and industrial complexes showed higher concentrations of mercury, zinc, arsenic, lead, copper, chromium and cadmium than sediments in locations unaffected by human activities (Mckee and Wolf, 1963). Fish from locations showing elevated concentrations of heavy metals in the sediments had higher concentrations of Hg, Zn, Cr, and Cu than fish from control locations (Mckee and Wolf, 1963). In the flesh of Michigan fish, only mercury approached concentrations presently considered by the U.S. Food and Drug Administration to be harmful to human health (Michigan Water Resources Commission, 1972).

Physical and Chemical Properties

Mercury is a metal which is liquid at room temperature and pressure. Mercury can exist in a wide variety of physical and chemical state. There are three forms of mercury: elemental, inorganic, and organic compounds (Goldwater, 1972). Metallic or elemental mercury in the atmosphere is the major pathway for global transport of mercury. Metallic mercury may be oxidized to inorganic divalent mercury, particularly in the presence of organic material, such as in the aquatic environment. Divalent inorganic mercury may, in turn, be methylated to dimethyl-mercury by anaerobic bacteria. If taken up by fish, dimethylmercury may eventually cycle through

higher levels of fish-eating animals or humans (Smith and Carson, 1981).

Sources of Exposure

Metals are redistributed naturally in the environment by both geologic and biologic cycles (Beijer and Jernelöv, 1986). For the geologic cycles, rainwater dissolves rocks and ores and physically transports material to streams and rivers, adding and deleting from adjacent soil, and eventually to the ocean to be precipitated as sediment or taken up in rainwater to be relocated elsewhere on earth (Li, 1981). The biologic cycles include bioconcentration by plants and animals and incorporation into food cycles (Friberg *et al*, 1986). One of the most significant effects of mercury pollution is that aquatic organisms can absorb and accumulate mercury in their tissues, leading to increasing concentrations in the food chain.

Methyl mercury is the most important form of mercury in terms of toxicity effects from environmental exposures (Goyer, 1991). Except in fish, mercury concentrations in food are very low, generally in the range of 5 to 20 μg/kg (NAS, 1977). Mercury concentrations in large carnivorous fish like pike and swordfish have been found to exceed 1,000 μg/kg (EPA, 1984). Therefore, consumption of fish by humans is the most important source of general population exposure to mercury. The mercury content in the fillet of lake trout in Lake Ontario was about 170 μg/kg in 1982 (Government of Canada, 1991). Between 70

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and 90 percent of the total mercury detected in fish muscle tissue is in the form of methyl mercury (WHO, 1976).

Absorption and Distribution

The physical and chemical properties of mercury allow them to gain entry into organisms, resist being metabolized or excreted, and to bioaccumulate (Gleason *et al.*, 1969). Elemental mercury volatilizes to mercury vapor at ambient air temperatures, and most human exposure is by inhalation. Mercury vapor readily diffuses across the alveolar membrane and is lipid soluble. Therefore, it has an affinity for red blood cells and the central nervous system (Roels *et al.*, 1985). For inorganic mercury and methylated mercury, gastrointestinal absorption is still the major route. Cells that are involved in the transport of mercury, such as gastrointestinal, liver, and renal tubular cells, have more chances to contact mercury than other cells (Thayer, 1984). Hence, liver and kidney are the primary organs for mercury accumulation (WHO, 1989c).

All forms of mercury can cross the placenta to the fetus in experimental animals. In rats, fetal uptake of elemental mercury has been shown to be 10 to 40 times higher than uptake after exposure to inorganic salts probably because of lipid solubility (Lauwerys, 1983).

Metabolism and Elimination

Within cells, mercury may bind to a variety of enzymes including those in microsomes and mitochondria and will

produce nonspecific cell injury or cell death (Luckey *et al.*, 1975). Mercury has a particular affinity for ligands containing sulfhydryl groups. In liver cells, methyl mercury forms soluble complexes with cysteine and glutathione, which are secreted in bile and reabsorbed from the gastrointestinal tract (Mottet *et al.*, 1985). Organomercurial diuretics are thought to be absorbed in the proximal tubule-binding specific receptor sites that inhibit sodium transport (Skerfving, 1974).

Biologic half-times are available for a limited number of mercury compounds. The biologic half-time for methyl mercury is about 70 days; the half-time for retaining salts of inorganic mercury is about 40 days (WHO, 1976). There are few studies on biologic half-time for elemental mercury or mercury vapor, but it also appears to be linear with a range of values from 35 to 90 days (WHO, 1976).

Toxicity

The major human health effects of mercury are neurotoxic effects in adults (Bakir *et al.*, 1973), and toxicity to fetuses of mothers exposed to methyl mercury during pregnancy (Cox *et al.*, 1989). The fetal brain is much more susceptible to methyl mercury than that of adults (Clarkson, 1987). This difference may be due to the fact that this toxicant depolarizes microtubules, thus interfering with cell division and migration, both of which are essential in the proper development of fetal brain (Paton and Allison, 1972; Clarkson,

1987). Clinical manifestations of neurotoxic effects in adults include parathesia, ataxia, vision and hearing loss, tremor, and finally, coma and death (Goyer, 1991).

Mercury has been shown to alter the reproductive function in rodents. A single intraperitoneal dose of 2 ppm mercuric chloride given to female mice 0.5 to 4.5 days before mating decreased the number of implants, living embryos, and increased embryo death (Suter, 1975). Lee and Dixon (1975) observed decreased fertility in male mice receiving a single intraperitoneal dose of 1 ppm mercuric chloride. The decreased fertility was observed between days 8 and 49 post-treatment and was associated with altered spermatogonia. Khera and Tabacova (1973) reported that daily oral gavage doses of 1, 2.5, and 5 mg methylmercuric chloride/kg body weight for 7 consecutive days administered to male rats caused a dose-related reduction in mean litter size. Reduced number of viable embryos was attributed to pre-implantation losses resulting from abnormal spermatogenesis due to mercury treatment of the parent male rat.

Statement of Problem

There have been many reported observations of adverse effects on reproductive performance of animals exposed to contaminants of the Great Lakes. Several epidemiological studies of the predators of the Great Lakes fish, including humans, have also showed less than optimum reproduction or declines in population in the past 20 years. Based on the physical and chemical properties and the toxicities of PCBs, TCDDs, HCB, aldrin, dieldrin, DDT, and mercury, the Great Lakes contaminants are a threat to this ecosystem.

Whereas much has been learned about the effects of single chemical on reproduction through high doses administered to animals or cultured cells, little is known of the effects of collective contaminants from Great Lakes on reproduction and other physiological functions. Long term, multi-generation, exposure to low concentration of environmental contaminants is an additional knowledge gap concerns most of the researchers today. Therefore, this study is designed to examine the long-term, low-dose exposure of the laboratory animals to Great Lakes carp.

Objective

This study was designed to study the effects of Great Lakes contaminants on reproductive performance. The specific aims were to determine: (1) the sperm quality of male mice and the oocyte fertilizing quality of female mice fed with fish diet prepared from Great Lakes carp; (2) the sexual maturation of the male offspring from the fish-fed mice; and (3) the effects of the treatments on hepatic vitamin A and E concentrations and the development of some other reproductive function related organs.

PHASE I STUDY

MATERIALS AND METHODS

Animals

Forty-eight 12-week-old CBA/CAJ female mice, purchased from Jackson Labs, Bar Harbor, ME, were bred with 16 CBA/CAJ male mice. The study was conducted beginning at October 16, 1991. The mice were housed in an environmentally controlled facility with a 12 hours light/12 hours dark cycle and at 21°C in the Food Science & Nutrition Building at Michigan State University (MSU). At birth, litters were assigned randomly into three treatment groups.

Treatment Diets

Each treatment group received one of the following diets formulated on a dry-matter basis:

C: 100% Lab Mouse Chow #5015 (control)

A: 47% farm-raised carp + 40% Lab Mouse Chow #5015 + 13% fish oil

G: 60% Great Lakes carp + 40% Lab Mouse Chow #5015

Lab Mouse Chow #5015 was purchased from Purina Mills Inc., St. Louis, MO. Farm-raised carp (*Cypinus carpio*) were purchased from the Billy Bland Fishery Inc., Taylor, Ark. Great Lakes carp were collected in August 1 and October 31,

1990 at the mouth of the Saginaw River with the assistance of the Fisheries Division of the Michigan Department of Natural Resources. Fish oil was kindly donated by Zapata Haynie Cop., Reedville, VA. The analysis of organochlorinated pesticides and PCBs in the fish oil is shown in Appendix 1.

Carp from both sources were ground, loaded into sausage casings and cooked at 170°F in the smoke house of the MSU Meat Lab. for 30 minutes to inactivate thiaminase (Gnaedinger and Krzeczkowski, 1966). All treatment diets were adjusted to contain 40 to 45% water. Major nutrients were balanced based on the Nutrient Requirements of Laboratory Animals (Appendix 2; NRC, 1978) and analyzed by Litchfield Analytical Services, Litchfield, MI. Biotin, folacin, niacin, riboflavin, thiamin, vitamin B_6, vitamin B_{12}, vitamin E, vitamin K_1, α-cellulose, and fish oil were used to balance the diet ration. Total vitamin A and E concentrations in the diets were analyzed by the Animal Health Diagnostic Lab., Nutrition Section, MSU. Total vitamin A concentration was calculated from the concentrations of retinol and retinyl palmitate. Additional mineral analyses were performed by the Animal Health Diagnostic Lab., Toxicology Section, MSU. Analysis of organochorinated pesticides and PCBs in the ground carp was performed before cooking and in the complete diets by the Aquatic Toxicology Lab., MSU.

Mouse Liver Analysis

Total vitamin A and E concentrations in mouse liver were analyzed by the Animal Health Diagnostic Lab., Nutrition Section, MSU. Analysis of organochorinated pesticides and PCBs in mouse liver was performed by the Aquatic Toxicology Lab., MSU.

Medium and Chemicals

Brinster's Medium for Ova Culture (BMOC-3), used for sperm capacitation and *in vitro* fertilization, was purchased from the Gibco Life Technologies, Gaithersburg, MD. BMOC-3 contains 189 mg/L $CaCl_2$, 356 mg/L KCl, 162 mg/L KH_2PO_4, 294 mg/L $MgSO_4$-$7H_2O$, 5546 mg/L NaCl, 2106 mg/L $NaHCO_3$, 5000 mg/L bovine serum albumin, 1000 mg/L D-glucose, 2253 mg/L D,L-sodium lactate, 56 mg/L sodium pyruvate, 3.3 mg/L streptomycin sulfate, and 3.3 mg/L penicillin potassium (Brinster, 1971). BMOC-3 was equilibrated in 5% CO_2 in air at 37°C overnight before use.

Folacin, niacin, riboflavin, thiamin, vitamin B_6, vitamin B_{12}, and α-cellulose were purchased from ICN Biomedicals, Inc., Costa Mesa, CA. Biotin and vitamin K_1 were purchased from Hoffman-LaRoche Chemical Division, Nutley, NJ; Vitamin E was obtained from Rhone-Poulenc Co., Atlanta, GA.

Pregnant mare serum gonadotropin (PMSG), human chorionic gonadotropin (hCG), bisBenzimide Hoechst No. 33258, and all other chemicals used in this study, except where indicated,

were purchased from Sigma Chem. Co., St. Louis, MO.

Experimental Design

Diets were provided *ad libitum* to dams (F-0) immediately after parturition and through lactation. Thus pups (F-1) were treated by the milk. Water was provided *ad libitum*. Pups (F-1) were culled to the size of 6 or 7 per litter. Body weights of pups were measured at days 1, 4, 7, 14 and 21. At 21 days of age, F-1 pups were weaned, sexed, and weighed, then housed in groups of 3 or 4 mice per cage. F-0 females were sacrificed by cervical dislocation. Liver weights, liver vitamin A and E concentrations of F-0 females were measured at weaning. The F-1 received their respective treatment diets continuously to termination.

The body weights of the F-1 males were recorded at 3, 4, 5, 6, 7, 8, 10, 14, 15, 21 and 23 weeks of age. Three livers from the F-1 male mice from each treatment were weighed at 7, 8 and 15 weeks of age. Liver vitamin A and E concentrations were analyzed at 19 and 32 weeks of age. At 34 weeks of age, five livers from each treatment were analyzed for organochorinated pesticides and PCBs.

Epididymal sperm concentration and motion were measured at 5, 6, 7, 8, 15, 23, 32 and 34 weeks of age. At 8 weeks of age, fertilizing ability of sperm from 3 F-1 males from each treatment were tested *in vitro* with oocytes from non-treated B6D2-F1 females.

At 7.5 weeks of age, 7 F-1 males were paired individually with previously non-treated mature CBA/CAJ females for 5 days. These females received the same diets as F-1 males during the pairing and were later housed individually. The bred females were continuously provided with treatment diets throughout gestation and lactation. At birth and weaning, litter size and viability of the offspring (F-2) were measured. Body weights of the F-2 pups were recorded at days 1, 4, 7, and 14 during lactation.

F-1 females, at 5 and 10 weeks of age, were superovulated (see Method for *in vitro* fertilization on p.51). The oocytes were inseminated *in vitro* with epididymal sperm from previously non-treated B6D2-F1 males. Ovulation and fertilization rates were recorded.

At 10 weeks of age, 7 F-1 females from each treatment group were bred with non-treated and proven fertile B6D2-F1 males for 5 days and sacrificed by cervical dislocation 10 days after the end of breeding period. The number of fetus was recorded.

Sperm Collection

Caudal epididymal sperm were collected for analysis and *in vitro* fertilization. Each pair of the epididymides was placed in the inner well of a Falcon Organ Tissue Culture Dish (60 × 15 mm style with a center well, #3037, Becton Dickinson Labware, Lincoln Park, NJ) containing 1.0 ml BMOC-3. The

outer well contained 3 ml BMOC-3 without bovine serum albumin (BSA). Epididymides were poked 30 times each with a 25 G needle to release the sperm. The dish, with the epididymides in the center well, was then incubated at 37°C, with 5% CO_2 in air and 100% humidity for one half-hour before recovered sperm concentration and motion analysis or use for *in vitro* insemination.

For recovered sperm concentration and motion analysis, 20 µl of the sperm suspension was placed on a CellSoft 20 µ chamber and examined under a CellSoft Video-Micrographic Computer-Assisted Semen Analyzer (CRYO Resources Inc., New York, NY). A minimum of 100 sperm cells were analyzed for each sample. Percentage of recovered sperm concentration, motile cells, velocity, linearity, amplitude of lateral head displacement and beat/cross frequency were assessed. See Appendix 3 for set up and definition of the parameters.

In Vitro Fertilization

Oocytes were collected from female mice superovulated by intraperitoneal injection of 10 IU PMSG followed 48 to 50 hours later by 10 IU hCG. Twelve to 14 hours after hCG injection, oviducts were removed and washed in 3 ml BMOC-3 without BSA in the outer well of a Falcon Organ Tissue Culture dish and then placed in the inner well with 1 ml BMOC-3. The ampulla portion of the oviducts were teased open to release the cumulus mass of oocytes. The cumulus masses were then

transferred to a second petri dish and placed in the center well containing 1 ml BMOC-3. Fifty μl of sperm suspension (1-3 x 10^7 cells/ml) that was incubated for one half-hour was then placed in the oocyte-containing well and incubated for 24 hours before examination of fertilization. The final sperm concentration at insemination was 0.5-1.5 x 10^6 cells/ml.

To determine fertilization, 100 μl of 370 μM bisBenzimide Hoechst No. 33258, a chromosome stain (Coming, 1975), was added to the petri dish and incubated for an additional 30 minutes. The Hoechst stain allows observation of the chromosome within nucleus. The oocytes were then washed by transferring them to 1 ml fresh BMOC-3 and transferred again onto a microscope slide. Oocytes/embryos were classified on a Nikon Optiphot microscope equipped with a 100 W mercury bulb, 365/10 nm excitation filter, 400 nm dichroic mirror, and 400 nm barrier filter. Two-cell embryos with a nucleus in each cell were classified as fertilized. Zygotes with one cell and two pronuclei were also classified as fertilized. Oocytes containing only one cell and one nucleus were classified as non-fertilized. Fragmented oocytes or those that lost their cytoplasm were classified as degenerated and non-fertilized.

Statistical Analysis

Data, except the superovulation rate, were analyzed using the Statistical Analysis System (SAS Institute, Inc., Cary,

NC). The effects of treatments were determined by one-way analysis of variance (one-way ANOVA). Significant differences among treatment groups were determined by least significant differences (LSD) for the comparisons of multiple means. For superovulation rate, Chi square (X^2) test was applied to determine if differences were significant. All statements regarding significance are based on p value < 0.05.

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RESULTS

Nutrient Contents of Treatment Diets

Diets were analyzed for nutrient, and organochorinated pesticides and PCBs. All diets contained about 45% water. Vitamin (Vit) E concentrations in both A and G fish diets were about 83% of that in diet C (Table 2). Vitamin A concentration in diet A was about the same as diet C; in diet G, however, it was only about two-thirds the concentration of diet C.

Mineral analysis of diets and mineral requirement for mice are shown in Table 3. Diets C and G contained similar amounts of major mineral with the exception of higher iron and lower sodium in diet G. Compared to diets C and G, diet A contained higher concentrations of calcium, iron, phosphorus, and aluminum.

The organochlorinated pesticides and PCBs in Great Lakes and Arkansas carp before cooking and in the treatment diets are shown in Table 4. The PCB concentrations in the Great Lakes carp before cooking was 7.12 ppm, whereas the amount in Arkansas carp was non-detectable (< 1 ppb). The diet G contained 2.54 ppm PCBs. Dieldrin, *p,p'*-DDE and *o,p'*-DDD concentrations were 7.14, 50.44, and 97.07 ppb in diet G and 1.79, 2.21, and 3.01 ppb in diet A, respectively.

Table 2. Nutrient analysis of treatment diets[a].

Nutrients[b]	Diet C	Diet A	Diet G
Fat, %	11.15	22.45	25.25
Crude protein, %	18.75	30.56	31.88
Crude fiber, %	2.00	2.50	2.40
Calcium, %	0.85	2.21	1.20
Phosphorus, %	0.59	1.25	0.86
Potassium, %	0.78	0.58	0.67
Magnesium, %	0.16	0.13	0.12
Sodium, %	0.47	0.38	0.32
Selenium, ppm	0.15	0.56	0.98
Iron, ppm	181.00	449.00	132.00
Manganese, ppm	130.00	128.00	59.00
Copper, ppm	17.00	12.00	11.00
Zinc, ppm	128.00	84.00	183.00
Ash, %	5.60	9.90	5.90
Vitamin A (IU/kg)	45331	42709	30636
Vitamin E (IU/kg)	52.06	42.85	44.03

[a] The nutrient analysis was determined by Litchfield Analytical Services, Litchfield, MI. Selenium and Vitamin A and E analyses were determined by Dr. Stowe's lab., the Animal Health Diagnostic Lab., Nutrition Section, MSU.

[b] Data expressed on dry-matter basis.

Table 3. Mineral analysis of three treatment diets and mineral requirement for mice[a].

Minerals[b]	Diet C	Diet A[c]	Diet G[c]	Requirement[d]
B	7.00	3.16	3.36	NS[e]
Ba	4.72	66.0	4.25	NS
Ca	8010	21700	8040	4000
Cu	17.0	10.8	10.0	4.5
Co	0.520	0.487	<0.200	NS
Fe	68.3	291	290	25
Mg	1610	1220	1020	500
Mn	129	147	53.2	45
Mo	1.91	0.678	0.791	NS
P	5310	12400	7160	4000
Zn	127	82.8	170	30
Al	155	769	114	NS
Sb	<2.00	<2.00	<2.00	NS
As	<1.00	<1.00	<1.00	NS
Cr	1.15	1.45	1.00	2
Cd	<0.200	<0.200	<0.200	NS
Hg	<4.00	<4.00	<4.00	NS
Pb	<1.00	<1.00	<1.00	NS
Se	<8.00	<8.00	<8.00	NS
Tl	<5.00	<5.00	<5.00	NS
Na	5190	4170	3760	NS
K	5650	4470	5310	2000

[a] The mineral analysis was determined by Dr. Braselton's lab., the Animal Health Diagnostic Lab., Toxicology Section, MSU.
[b] Data expressed as ppm on dry-matter basis.
[c] Diets A and G contained no visible bone.
[d] Mineral requirement for mice is based on Nutrient Requirements of Laboratory Animals (Appendix 2; NRC, 1978).
[e] Data are not available.

Table 4. Organochlorinated pesticides and PCBs in raw carp and mouse diets.[a,b]

	Arkansas carp		Great Lakes carp	
	Before cooking	Diet A	Before cooking	Diet G
PCBs (total)	<IDL	<IDL	7120	2542
Endosulfan I	<IDL	<MDL	<MDL	<IDL
Dieldrin	<MDL	1.79	<MDL	7.14
Endrin	<IDL	<IDL	<IDL	<IDL
Endosulfan II	<IDL	<MDL	<IDL	<MDL
Methoxychlor	<IDL	<MDL	<IDL	<IDL
Heptachlor	1.58	<IDL	1.54	<IDL
Heptachlor epoxide	<MDL	<MDL	<MDL	<MDL
Lindane	1.81	<MDL	<MDL	2.62
Oxychlordane	<IDL	<MDL	<MDL	<MDL
Gama-chlordane	1.11	<MDL	3.01	1.31
Alpha-chlordane	<IDL	<MDL	2.73	<IDL
t-nonachlor	<MDL	<MDL	10.65	18.46
p,p'-DDT	<IDL	<MDL	<MDL	2.75
p,p'-DDE	38.6	2.21	377.13	50.44
o,p'-DDE	<IDL	1.32	<MDL	7.19
p,p'-DDD	1.24	<MDL	6.13	6.25
o,p'-DDD	1.28	3.01	45.95	97.07

[a] The organochlorinated pesticides and PCBs analyses were determined by Dr. Giesy's lab., the Aquatic Toxicology Lab., MSU.

[b] Data expressed as ppb on wet-weight basis. Pesticides that were not detected by gas chromatography are labeled <IDL (instrument detective limit). Pesticide concentrations in the sample that are < 1 ppb are marked <MDL (method detective limit). Minimum detection level is 1 ppb and the quantification limit is 5 ppb.

F-0 Dams

F-0 dams were sacrificed when the offspring were weaned at 3 weeks postpartum. There was no difference in body weights (BW) of F-0 dams among all three groups (Figure 1; Appendix 4). Livers from the group G females were heavier than those in group A, which in turn were heavier than those in group C (Figure 1). In both fish treatment groups G and A, liver vitamin A concentrations were about 50% lower, and liver vitamin E concentrations were about 80% lower than those in group C (Figure 2; Appendix 5). Because there was only 1 mouse per treatment group for this measurement, no statistical analysis is shown here. There was no difference in 21-day body weights of F-1 among all treatment groups (Figure 3; Appendix 6).

F-1 Males

After weaning, BW of F-1 males was not significantly different in groups G and C (Figure 3). From 14 weeks of age, males in group A, however, had significantly lower body weights than those in groups G and C (Figure 3). Liver weights (LW) were not different at 7 and 8 weeks of age among all treatment groups. At 15 weeks, LW of F-1 males in treatment A were less than males in treatment C and G (Figure 4; Appendix 7). The LW/BW ratios, however, were greater in groups A and G than in group C at week 7 and 8, and similar mong all treatment groups at 15 weeks of age (Figure 5;

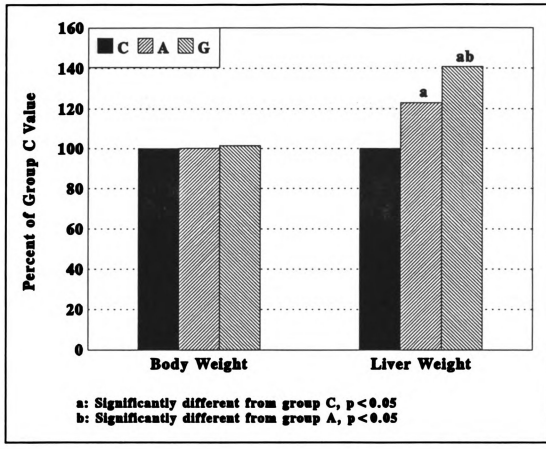

Figure 1. Body and liver weights of F-0 lactating mice. F-0 females were provided with treatment diets immediately after parturition and through lactation.

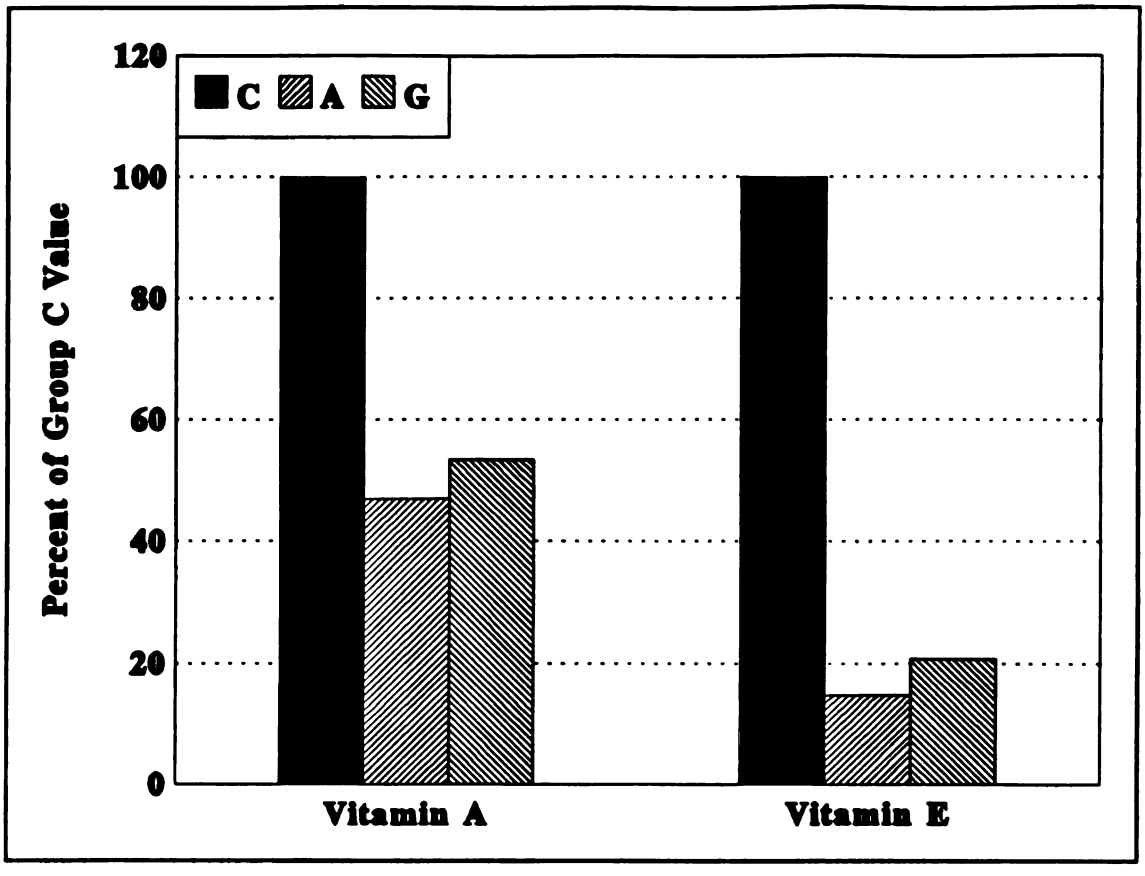

Figure 2. Liver vitamin A and E concentrations of lactating F-0 females. F-0 females were provided with treatment diets immediately after parturition and through lactation.

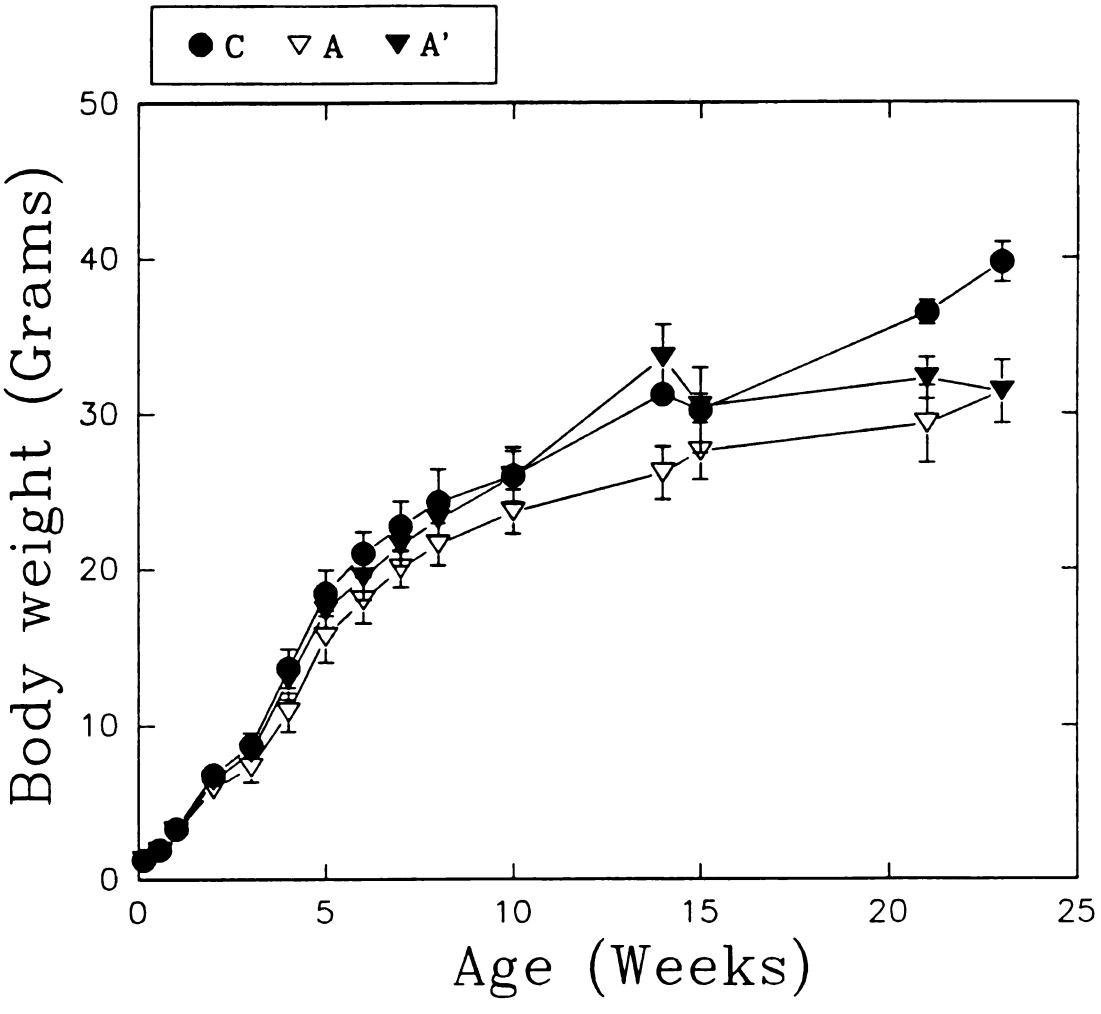

Figure 3. Body weights of F-1 male mice in Phase I from birth to 23 weeks of age. There was no significant difference in body weights among treatment groups prior to 10 weeks of age. At weeks 14, 21, and 23, body weights of Group C were significantly larger than those of Group A and G.

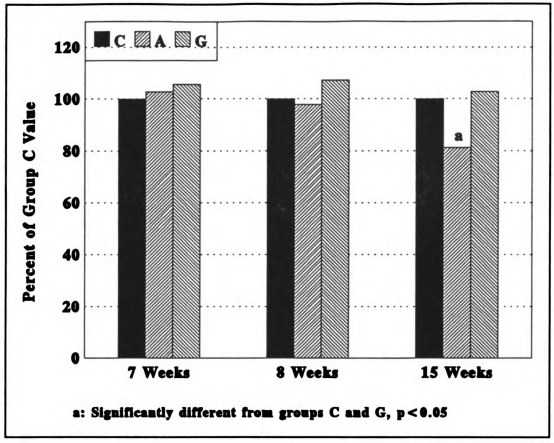

Figure 4. Liver weights of F-1 males at 7, 8, and 15 weeks of age.

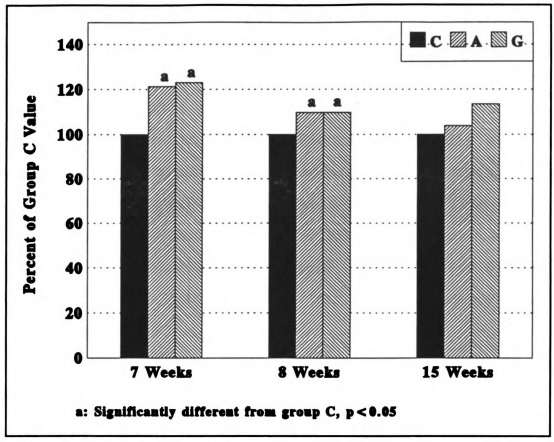

Figure 5. Liver weight over body weight ratios of F-1 males at 7, 8, and 15 weeks of age.

Appendix 7). There was no significant difference in testis weights of F-1 males among all treatment groups (Appendix 7).

Similar to the F-0 dams, liver vitamin A and E concentrations of F-1 males were significantly lower in group G and group A than in group C (Figure 6; Appendix 8). The concentrations of organochlorinated pesticides and PCBs in the liver of F-1 males are shown in Table 5. Livers from group G had the highest concentrations of PCBs (4.2 ppm), dieldrin (9.29 ppb), oxychlordane (18.3 ppb), *p,p'*-DDE (49.59 ppb), chlordane (17.83 ppb) and HCB (13.44 ppb) among the 3 treatment groups.

For the epididymal sperm analysis, no sperm cells were observed at 5 weeks of age in samples from any treatment groups (Figure 7; Appendix 9). A steady increase in sperm concentration was observed from 6 to 8 weeks of age. The concentrations reached a plateau at 8 weeks of age. No apparent difference in sperm concentrations or motion parameters were observed among the treatment groups at the ages examined (Appendix 10). *In vitro* fertilizing ability of the sperm from F-1 males at 7.5 weeks of age did not differ among all treatment groups (Appendix 11).

In the breeding study, fecundity and litter size were not significantly different among all treatment groups (Figure 8). Viability of the pups (F-2) at 7 days of age in groups G and A, was lower than that in group C (Figure 8); the viabilities in groups G, A, and C were 45%, 12%, and 94%, respectively.

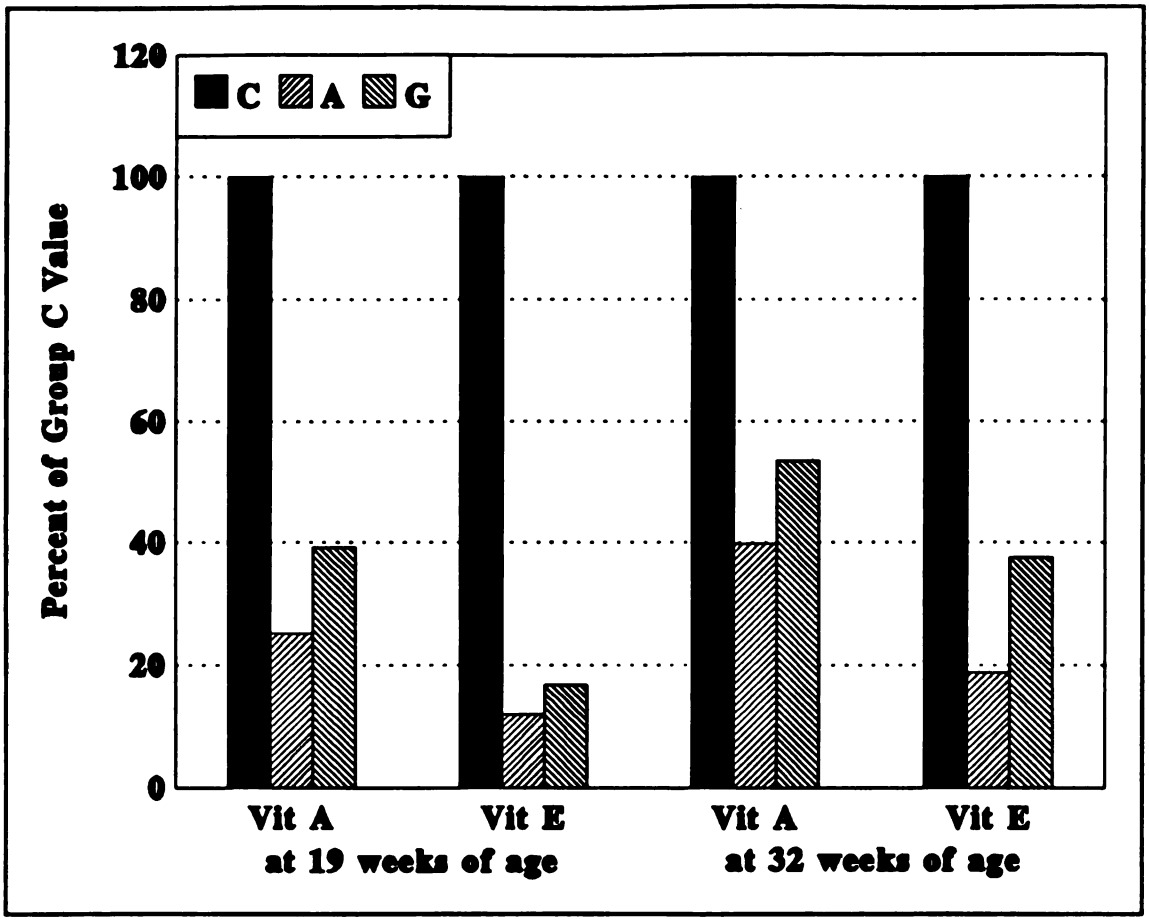

Figure 6. Liver vitamin A and E concentrations in F-1 males.

Table 5. Organochlorinated pesticides and PCB residues in F-1 male liver[a,b,c].

	Group C	Group A	Group G
PCBs	<IDL	150	4200
Endosulfan I	<IDL	<IDL	<IDL
Endosulfan II	<IDL	0.32	0.91
Aldrin	<IDL	<IDL	<IDL
Dieldrin	1.04	2.06	9.29
Endrin	<IDL	<IDL	<IDL
Methoxychlor	<IDL	0.45	<IDL
Heptachlor epoxide	1.31	0.87	<IDL
Oxychlordane	<IDL	1.52	18.30
Y-chlordane	<IDL	<IDL	0.61
A-chlordane	0.34	1.72	17.83
t-nonachlor	<IDL	<IDL	0.25
Hexachlorobenzene	0.22	0.59	13.44
Heptachlor	<IDL	<IDL	<IDL
p,p'-DDT	<IDL	<IDL	<IDL
o,p'-DDD	0.21	<IDL	<IDL
p,p'-DDD	<IDL	<IDL	0.51
o,p'-DDE	<IDL	0.30	1.89
p,p'-DDE	0.47	2.85	49.59

[a] The organochlorinated pesticides and PCBs analyses were determined by Dr. Giesy's lab., the Aquatic Toxicology Lab., MSU.

[b] Data expressed as ppb on wet-weight basis. The water content in three diets was similar and was 45%. Pesticides that were not detected by the gas chromatography are labeled <IDL (instrument detective limit). Pesticide concentration < 0.2 ppb and PCB concentration < 0.03 ppm in samples are below the quantification limit.

[c] F-1 male mice were 239 days old (32 weeks).

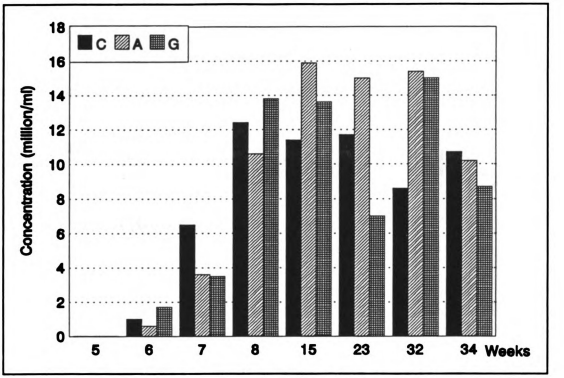

Figure 7. Caudal epididymal sperm concentrations of F-1 males at 5, 6, 7, 8, 15, 23, 32, and 34 weeks of age. Sperm suspension was collected as follows: both of the epididymides were placed in a Falcon Organ Tissue Culture Dish (60 x 15 mm, Becton Dickinson Labware) and poked with a 25 G needle to release the sperm into 1.0 ml BMOC-3 medium. After a 30-min incubation at 37°C, 5% CO_2 in air and 100% humidity, 20 μl of the sperm suspensions were placed on a CellSoft 20μ-chamber and examined under a CellSoft Video-micrographic Computer-Assisted Semen Analyzer (CRYO Resources Inc., New York, NY). A minimum of 100 sperm cells were analyzed for each sample.

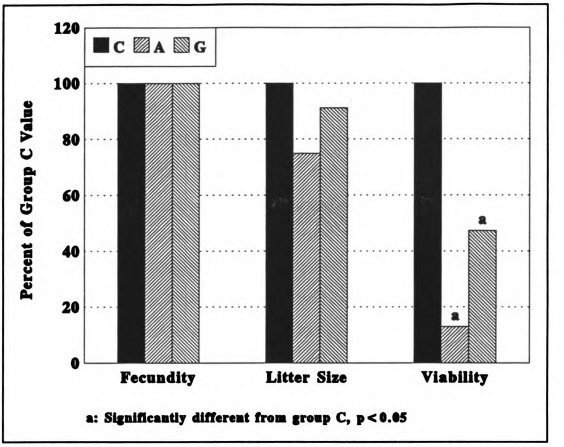

Figure 8. Reproductive performance of 52-day-old F-1 males.

F-1 Females

To test oocyte fertilizing ability, F-1 females at 5 and 10 weeks of age were superovulated and the oocytes were collected and inseminated *in vitro* with epididymal sperm from non-treated males. Significantly fewer females in groups A (4 of 6) and G (0 of 7) than in group C (7 of 8 females) responded to the superovulation treatment (Figure 9; Appendix 12). Fertilizing ability of oocytes collected from groups A did not differ C showed no difference *in vitro* (Appendix 12).

To study the reproductive performance, 10-week-old F-1 females were paired with proven fertile males for 5 days. Ten days after the breeding period, females were sacrificed to determine the number of fetuses. Groups A and G had about a 50% pregnancy rate; whereas, all 7 females in group C were pregnant (Figure 9). The number of fetuses per dam was not different among treatment groups (Figure 9).

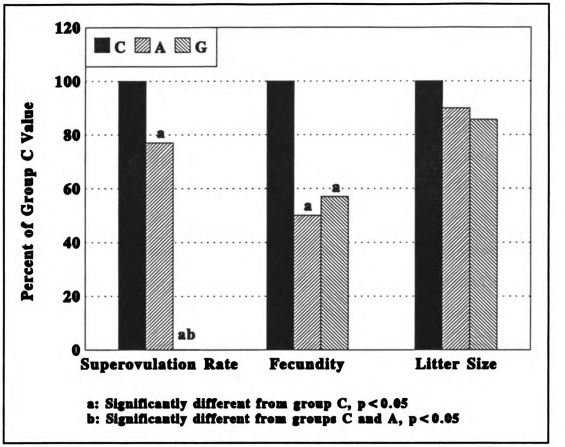

Figure 9. Reproductive performance of F-1 females. Superovulation rate refers to number of females ovulated/ number of females superovulated with PMSG/hCG. Fecundity refers to number of females carrying fetus 10 days after breeding period/ number of females bred.

DISCUSSION

The fish-containing diets were designed to contain the highest possible carp content and still maintain palatable texture for mice. Based on the results of a preliminary palatability study, 75% carp on a wet-weight basis was used to formulate the treatment diets. The carp content in the final diets was 60% on a dry-matter basis.

The PCB concentration was 7.12 ppm in the Great Lakes carp before cooking and 2.54 ppm in diet G containing 75% cooked Great Lakes carp (Table 4). Since diet G contained 75% cooked Great Lakes carp on a wet-weight basis, the PCB concentration in the 100% cooked Great Lakes carp was calculated to be about 3.39 ppm. The decrease in PCB concentration from 7.12 ppm to 3.39 ppm apparently was due to the cooking process.

Enlarged livers were observed in both fish treatment groups (Figures 1 and 5). In F-0 lactating mice, although liver weights from group A were less than those from group G, both were significantly heavier than those from group C (Figure 1). This change in liver weight, however, was not detected in F-1 males (Figure 4). Nevertheless, when data of F-1 males were expressed as LW/BW ratio, groups A and G showed significantly higher ratios than group C at 7 and 8 weeks of age, although this higher LW/BW ratio was less apparent in F-1 males at 15 weeks of age (Figure 5).

Increased liver weights were also observed in other studies where animals were exposed to PCBs, TCDD or other related compounds (Risebrough *et al.*, 1968; Fowler *et al.*, 1973; McConnell, 1980). This may relate to an increase in total liver lipid and triglyceride contents (Azaïs-Braesco *et al.*, 1990) or a proliferation of the smooth endoplasmic reticulum (Fowler *et al.*, 1973; Buchmann *et al.*, 1991). Since no detectable PCBs and less than 7 ppb DDT metabolites were observed in diet A (Table 4), the statement above does not explain the enlarged livers in group A mice observed in both F-0 dams and F-1 males. Therefore, ingestion of 60% fish diet on dry matter basis will cause liver enlargement in mice.

Decreased liver vitamin (Vit) A concentration in PBBs- and PCBs-exposed animals was reported previously (Spear *et al.*, 1988; Mercier *et al.*, 1990). PCBs were shown to alter Vit A metabolism and distribution, leading to a reduction in Vit A storage in the liver. When the liver Vit A storage was decreased, the storage form of Vit A, retinyl esters, which normally represent 95% of the hepatic Vit A contents, was diminished (Mercier *et al.*, 1990). Such transformation of retinyl esters to retinol (mobile form) should involve enhanced hydrolysis of retinyl esters into free retinol (Mentlein and Heymann, 1987). In 1984, retinyl palmitate hydrolase was the only enzyme shown to be active in the reaction *in vivo* (Goodman and Blaner, 1984). More recently, another enzyme, namely retinyl ester hydrolase, was also found

to possess the characteristics of retinyl esters hydrolysis in male Wistar rat liver homogenate and microsomes (Mercier *et al.*, 1990). Both enzymes, belonging to the cytochrome P-450 system, could be affected by PCBs, DDT or TCDD, and may have had the potential to change hepatic Vit A storage (Mercier *et al.*, 1990).

Because of the higher concentrations of PCB or DDT metabolites in diet G than in diet A, it is reasonable to hypothesize that mice from group G would have lower liver Vit A concentrations. However, F-0 dams and F-1 males in group A, which consumed a diet containing no detectable PCBs and only trace amounts of other organochlorinated pesticide metabolites, had lower liver Vit A than those of group G (Figures 2 and 6). Therefore, effects of liver Vit A discussed above does not explain the lower Vit A concentrations in group A livers than those in groups C and G. One possible cause might be the high fish-oil content of the fish diets. Fish oil, highly unsaturated fatty acid, if undergoing active oxidative rancidity, could cause Vit E destruction (Scott *et al.*, 1982). Under continuously oxidative stress, once the majority of Vit E was depleted, Vit A will soon be attacked by newly generated radicals (see Phase II-Discussion).

The concentrations of organochlorinated pesticides and PCB residues in F-1 male livers were directly related to those in their respective diets (Table 5). In both fish eating

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groups, the liver residues of PCBs, dieldrin, oxychlordane, A-chlordane, and p,p'-DDE were higher than those of diet C.

In this study, caudal epididymal sperm concentrations and sperm motion were not significantly different among the three groups at all ages measured. These findings were similar to the work of Sager *et al.* (1991), who observed no differences in caudal sperm reserves, sperm production, epididymal sperm morphology, and epididymal sperm motility in male Sprague-Dawley rats when exposed to Aroclor 1254 at doses of 8, 16, 32 or 64 μg/g of body weight on days 1, 3, 5, 7, and 9 during lactation. However, they did report abnormal sperm function that was tested by breeding with non-treated females followed by embryo recovery. Increases in the percentage of abnormal embryos from pronuclear to blastocyst stages were observed in the 32 and 64 μg/g groups. Based on the *in vitro* fertilization (IVF) assay, I did not observe any difference in the percentage of fertilized oocytes, nor did I observe any difference in litter size in the breeding study.

The survival rate of F-2 pups from both carp-eating groups was significantly lower than that of the lab chow controls. Nutrient differences between lab mouse chow and both fish diets should be responsible for this observation. Survival rates for F-2 and body weights of F-1 were the lowest in those mice receiving diet A. The reason for these observations is not clear. Since mineral concentrations, such as calcium, phosphorus, iron, manganese and zinc were the

major differences between diets A and G, high minerals in diet A should account for the effects observed in the animals received diet A.

No F-1 females in group G responded to the PMSG/hCG-inducing superovulation (Figure 9). Other studies have demonstrated that PCBs could interfere with ovulation in two ways. First, PCBs accumulation would disturb the normal reproductive development and ovulation by stimulating the P-450 enzyme system, which metabolizes the steroid hormones leading to a change of the concentration of these substances in the body during the critical neonatal period when the young suckle PCB-contaminated milk (Lucier *et al.*, 1977). Second, PCBs and DDT may interfere with ovulation by their estrogenic effects (Fuller and Hobson, 1986). The weak estrogenic activity of PCBs and other pesticides could affect endocrine function by their competitive affinities for estrogen receptors within reproductive organs and, furthermore, by disrupting the feedback control between the reproductive organs (or other endocrine organs) and hypothalamus. Therefore, I hypothesize that PCBs and other pesticides in diet G were responsible for the failure of ovulation in group G.

The F-1 females were bred with previously non-treated and proven fertile males. The results, however, did not support the previous hypothesis that PCBs and other pesticides in the diet G were responsible for the failure of ovulation in group

G. In the study of reproductive performance of F-1 females, over 50% of the group G females were pregnant. One possible reason for failure to collect any oocyte from group G mice may be that Great Lakes contaminants changed the timing of ovulation after PMSG/hCG injection. Thus, no oocytes could be collected from the oviduct at the expected time.

Pregnant rate of F-1 females in group G were much lower than those of the control group (Figure 9). Similar findings were also reported in other PCB studies. Kihlstrom *et al.* (1975) administered a PCB mixture to pregnant mice on the day of delivery and repeated this 3 times weekly. The reproductive capacity of F-1 males was measured by the frequency of implantation in F-1 males mated females that were non-treated previously. The reproductive capacity was 94% in the control F-1 males, and was about 20% lower than that in control males in the PCB-treated males. Örberg and Kihlström (1973) reported prolonged estrus and decreased reproductive capacity after administration of PCBs (Chlophen-A-60) to mice. On the contrary, Rönnbäck (1991) found no indication of altered litter size in neonatally exposed females mice (15 ppm tetrachlorobiphenyls/day). The treated females showed no indication of developmental toxicity: no gross malformations, no obvious changes in size, and no increased mortality were observed during the lactation period. In this study, not only those females in group G had lower fecundity, but also those in group A had similar results. It is apparently that there

were critical factors other than the Great Lakes contaminants causing these adverse effects. Again, ingestion 60% fish diet may be the reason for low fecundity observed in both fish eating groups.

Recently, a study of delayed first ovulation by a diet containing high percentage of fish oil was published (Zhang *et al.*, 1992). This impaired gonadal development, retarded or prevented the first ovulation in female rats by 21% fish oil in the diet was not due to nutritional imbalance. It was believed that eicosapentaenoic acid, a fatty acid in the fish oil, could compete for cyclooxygenase and reduce the synthesis of dienoic prostanoids including prostaglandin E_2 which plays an important role in the estrogen-stimulated release of hypothalamic GnRH. In this study, the percentage of fish oil in diets A and G were 22.45% and 25.25%, respectively. Therefore, fish oil could be a possible factor causing the low reproductive performance in groups A and G.

PHASE II STUDY

In the Phase I Study, the results of Vit A and E concentrations in mouse livers showed that Vit A concentrations in groups A and G were about 50% or lower than that in group C; whereas Vit E concentrations in groups A and G were less than 20% of that in group C (Figures 2 and 6). Because decreases in Vit A and E concentrations in the liver were observed in both groups A and G, factors other than the Great Lakes contaminants were thought to be causing the altered metabolism and/or distribution of the vitamins.

The hypothesis was that oxidative stress generated from the fish oil exhausted Vit E which is an antioxidative agent. Following the depletion of Vit E, Vit A would also be depleted by the oxidative stress. This may occur both during feed storage and *in vivo*.

The objectives of this Phase II study were to determine: 1) whether increased dietary Vit E concentration will elevate the low hepatic Vit E concentration, and 2) whether increased dietary Vit E concentration will in turn raise the hepatic Vit A concentration in both groups A and G .

MATERIALS AND METHODS

Animals

Nine 81-day-old F-1 male mice (3 from each group) from the first phase were used in the second phase of study.

Treatment Diets

Two new diets, A+ and G+, which contained Vit E concentrations 5 times greater than those of the original diets (A and G) were made for this study. One hundred and five μg/kg Vitamin E, which was 4 times the concentration of previous diets, was added to diets A and G to make the final Vit E concentrations 5 times greater than the original diets. Group C was under the same treatment diet (diet C) as in Phase I study.

Experimental Design

There were 3 treatment groups, C, A+, and G+, in this study. Three mice each from the previous groups A and G were fed the high Vit E diets (diets A+ and G+); whereas, 3 mice from group C were fed diet C continuously. Animals were sacrificed at 10, 30, and 156 days after consuming the high Vit E diets. Liver vitamin A and E concentrations were analyzed as in Phase I.

RESULTS

Liver Vit E concentrations in both groups A+ and G+ were higher than those of group C after being supplied with high Vit E diets for 10 days and remained higher at day 30 and day 156 (Figure 10; Appendix 13).

The liver Vit A concentrations in both groups A+ and G+ reached about 60% of those in the control after the mice were fed diets with high Vit E for 10 days (Figure 10). After 30 and 156 days of the high Vit E supplement, liver Vit A concentrations in groups A+ and G+ were higher than those of group C. When the data were expressed as a percentage of group C, the liver Vit A concentrations in group A+ were 54% higher after 30 days and 82% higher after 156 days of the Vit E supplement. Concentrations of liver Vit A in group G+ were about the same as those in group C after 30 days and 47% higher after 156 days following the treatment.

Although no significant difference in LW/BW ratio among treatment groups, same as F-1 males in Phase I study, there was still a trend that mice in group G had larger LW (Appendix 14).

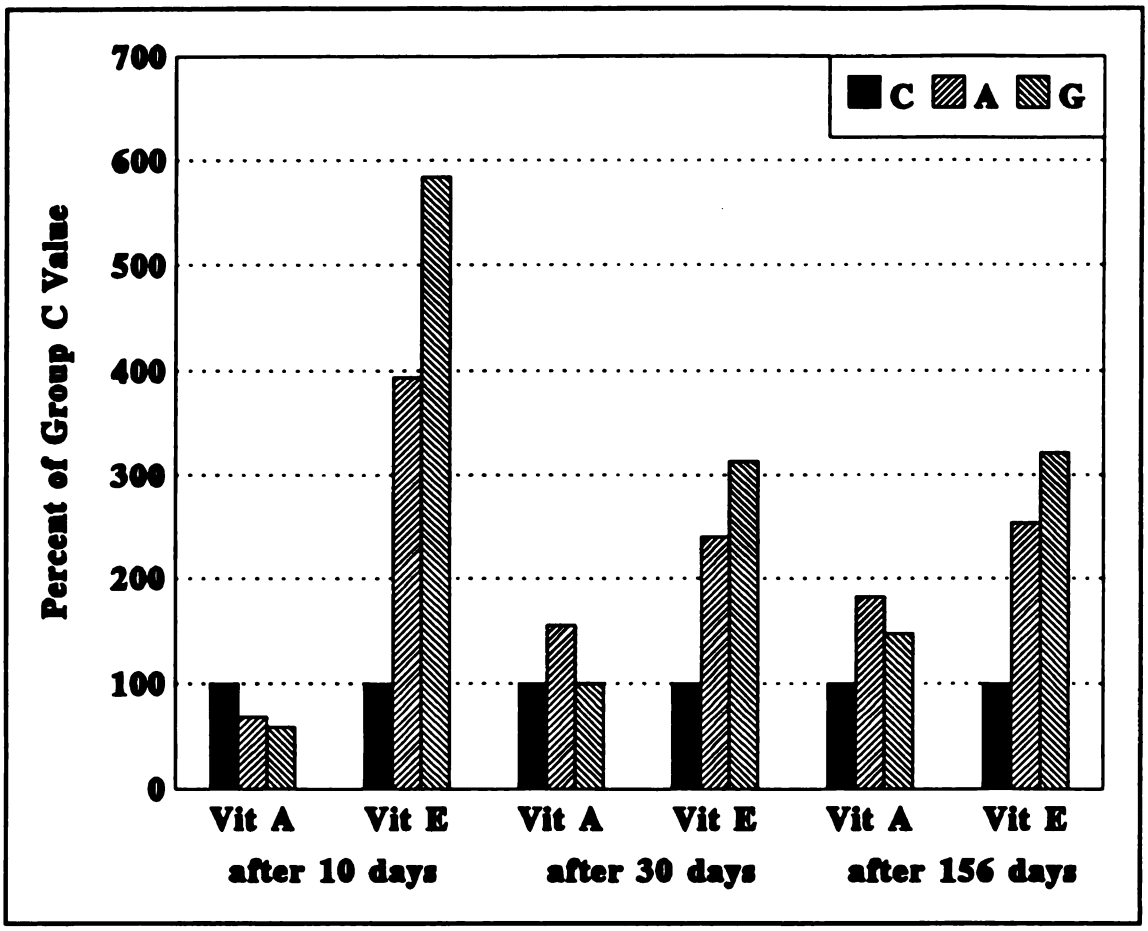

Figure 10. Liver vitamin A and E concentrations of F-1 males after high dietary vitamin E supplementation.

DISCUSSION

The NRC (1978) requirement for vitamin (Vit) E for mice is 20 IU/kg in the diet. However, Vit E requirements are exceedingly difficult to determine because of their interrelationships with other dietary factors, such as polyunsaturated fatty acid (PUFA), other antioxidants, sulfur amino acids and selenium (Se) (Church and Pond, 1985).

Vitamin E is very unstable; its oxidation is increased by the presence of PUFA, oxidizing agents, and trace minerals (eg. Fe), and decreased with increasing levels of fat-soluble antioxidants, sulfur amino acids, or Se (Church and Pond, 1985). Requirements of both Vit E and Se are greatly dependent on each other's dietary concentrations. Low Se content in the diet was believed to cause Vit E deficiency (Trapp *et al.*, 1970). In the present study, Se concentrations were the highest in diet G and lowest in diet C (0.15, 0.56, and 0.98 ppm in diets C, A, and G, respectively) (Table 2). Therefore, the possibility of lower liver Vit E concentrations caused by low Se concentrations in the diet can be ruled out.

The high concentrations of PUFA found in unsaturated oils such as cod-liver oil, corn oil, soybean oil, sunflower-seed oil and linseed oil can increase Vit E requirements. This is especially true if these oils are allowed to undergo oxidative rancidity in the diet or are in the process of peroxidation

after consumption by the animal (Scott *et al.*, 1982). If a diet becomes completely rancid before ingestion, the only damage done is the destruction of the Vit E present in the oil and in the feed containing the rancidifying oil. But if a diet undergo active oxidative rancidity after consumption, it will cause destruction of body stores of Vit E (Scott *et al.*, 1982).

Dietary supplementation of Vit E not only raised the hepatic Vit E concentrations in the mice, but also elevated the hepatic Vit A concentrations (Figure 10). Since dietary Vit E can protect or spare body supplies of oxidizable materials, such as Vit A and C, by interruption of fat peroxidation, it is reasonable to deduce that the lower liver Vit A observed in the previous study was mostly caused by the oxidative rancidity. After higher Vit E was supplied, it protected Vit A (and perhaps many other nutrients) from the PUFA-generated radicals.

Phase III Study

After reviewing the nutrient contents in the 3 treatment diets of Phase I (Table 2), it was thought that the higher calcium, phosphorus, iron, manganese and lower zinc concentrations in diet A than those in diet G may have caused the lower body weights of F-1 and decreased viability in F-2 pups of group A. In trying to make diets A and G nutrients more alike, two diets, A' and G', were designed to eliminate the differences described above.

Another factor that should be addressed here is that, in the Phase I study, CBA/CAJ female mice did not ideally respond to PMSG/hCG stimulation at the expected time. The average number of oocytes collected by superovulation treatment was about 6 per mouse in non-treated CBA/CAJ females; whereas, in our experience, collecting more than 30 oocytes in B6D2-F1 females was common (B6D2-F1 is named after its maternal strain C57BL/6J and paternal strain DBA/2J). Although there were 87% of the females in group C responsed to superovulation during Phase I (Appendix 11), more than 50% ovulated only 3 to 4 oocytes. At this point, it is not certain whether the 0% ovulation rate in the group G females during Phase I (Figure 9) showed truly biological significance or not. Therefore, it was decided to change the mouse strain from CBA/CAJ to B6D2-F1 during this experiment phase.

MATERIALS AND METHODS

Animals

Thirty C57BL/6J female mice (Jackson Labs, Bar Harbor, ME) were bred with 15 DBA/2J male mice. One male was randomly caged together with 2 females. The mice were kept in the same facilities and under the conditions as described for Phase I. When the females were found pregnant, they were randomly divided into 5 treatment groups-- C, A, A', G and G'.

Treatment Diets

Five treatment groups, C, A, A', G, and G', were included in this phase of the study. Each group received one of the following diets formulated on a dry-matter basis:

C : 100% Lab Mouse Chow #5015 (control)

A : 47% farm-raised carp + 40% Lab Mouse Chow #5015 + 13% fish oil

A': 44.2% farm-raised carp + 40% Lab Mouse Chow #5015 + 15.8% fish oil + 100 ppm Zn

G : 60% Great Lakes carp + 40% Lab Mouse Chow #5015

G': 58.65% Great Lakes carp + 40% Lab Mouse Chow #5015 + 0.95% Ca + 0.40% P + 317 ppm Fe + 70 ppm Mn.

The mineral sources of Zn, P, Fe and Mn were from $ZnSO_4$, $CaHPO_4$, $FeSO_4$, and $MnSO_4$, respectively. Calcium, however, was supplied from 2 sources: $CaCO_3$ and $CaHPO_4$. All 5 mineral

supplements were purchased from ICN. Lab Mouse Chow #5015, fish oil, farm-raised carp and Great Lakes carp used in this trial were the same as in Phase I. The diets were prepared in the same manner as in Phase I.

Medium and Chemicals

BMOC-3 (for sperm capacitation and *in vitro* fertilization), PMSG, hCG, and Hoechst #33258 bisBenzimide stain were described previously in the **Medium and Chemicals** section of **Materials and Methods** of the Phase I study (pp. 48).

Experimental Design

The treatment diets were provided immediately after the dams (F-0) gave birth and through lactation. Body weights and liver weights of the F-0 females were measured at 21 days after parturition.

At 21 days of age, F-1 mice (B6D2-F1) were weaned, sexed, weighed, and then housed in groups of 3 or 4 mice per cage. The offspring (F-1) received their respective treatment diets continuously to termination.

Body weights were recorded every week before 10 weeks of age, and every 5 weeks after 10 weeks of age. Liver weights, thymus weights and testis weights were measured at 8 and 11 weeks of age. Liver concentrations of vitamin A and E were analyzed at 8 weeks of age. Sperm concentration and motion

were measured at 8 and 11 weeks of age under the same procedures as in Phase I. Sperm fertilizing ability of F-1 males, at 8 weeks of age, was tested *in vitro* with oocytes from non-treated B6D2-F1 females.

At 8 weeks of age, 8 F-1 males were paired each with a previously non-treated mature B6D2-F1 female mouse for 5 days. The females, received the same treatment diet as the males when they were housed together, and when housed individually through gestation and lactation. Litter size, body weights, gestation index (survival rate to day 4) and lactation index (survival rate to day 21) of the offspring (F-2) were recorded. The dams were sacrificed by cervical dislocation at 21 day postpartum to measure body and liver weights.

F-2 mice, at 21 days of age, were weaned, sexed, weighed, and then housed in groups of 3 or 4 mice per cage. The F-2 males received the same diet as dam continuously to termination. Body weights were recorded every week until termination (8 weeks of age). Liver vitamin A and E concentrations were measured at 6 weeks of age. Liver weights, thymus weights, testis weights and sperm concentration and motion were measured at 5, 6, 7, and 8 weeks of age. Sperm fertilizing ability of the F-2 males, at 8 weeks of age, was tested *in vitro*. At 7 weeks of age, 6 F-2 males were paired individually with a previously non-treated mature B6D2-F1 female mouse. Litter size and viability index (survival to day 7) of the offspring (F-3) were recorded.

F-1 females were fed their treatment diets the same as the F-1 males. At 52 to 58 days of age, 15 females from each treatment groups were superovulated by PMSG/hCG. Ovulation rates and *in vitro* fertilization rates were recorded. Body weights, liver weights and thymus weights were also recorded.

F-2 females were fed their treatment diets under the same conditions as the others. Between 31 to 44 days of age, they were superovulated. Ovulation and *in vitro* fertilization rate were recorded. Body weights, liver weights and thymus weights were recorded. Liver vitamin A and E concentrations were analyzed at 44 days of age.

In vitro Fertilization

The procedure of *in vitro* fertilization was described previously in the **Materials and Methods** section of the Phase I study (pp. 51).

Statistical Analysis

Data were analyzed as previously described in the Phase I study (pp. 52).

RESULTS

Nutrient Contents of Treatment Diets

The nutrient contents for diets C, A, A', G and G' are shown in Table 6. The nutrients in diets A' and G' listed in Table 6 were based on the calculation from diets A and G, and their respective additives.

F-0 Dams

After 21 days of lactation, there was no difference in body weights (BW) among the F-0 lactating dams (Figure 11; Appendix 15). Although the liver weights (LW) of the mice in groups G, G' and A' were heavier than those in groups A and C, the differences were not statistically significant ($p = 0.10$). When data were expressed as LW/BW ratios, groups G, G' and A' were significantly larger than those of groups C and A.

F-1 Males

After weaning, F-1 males were fed their respective treatment diets continuously to termination. Although the BW of F-1 males in groups A, A' and G' were constantly smaller than those of group C and G, the differences were not significant until after 15 weeks (Figure 12; Appendix 16). The group C mice were the heaviest; while those in groups A and A' were the lightest. No differences in the BW were observed between groups G and G' at 15 and 20 weeks of age.

Table 6. Nutrient contents of the treatment diets[a].

Nutrients[b]		C	A	A'	G	G'
Dry Matter	%	55.00	53.96	53.96	54.82	54.82
Fat	%	11.15	22.45	25.25	25.25	25.25
Crude Protein	%	18.75	30.56	30.56	31.88	31.88
Crude Fiber	%	2.00	2.50	2.50	2.40	2.40
Ash	%	5.60	9.90	9.90	5.90	9.90
Calcium	%	0.85	2.21	2.21	1.25	2.20
Phosphorus	%	0.59	1.25	1.25	0.86	1.26
Potassium	%	0.78	0.58	0.58	0.67	0.67
Magnesium	%	0.16	0.13	0.13	0.12	0.12
Sodium	%	0.47	0.38	0.38	0.32	0.32
Iron	ppm	181.00	449.00	449.00	132.00	449.00
Manganese	ppm	130.00	128.00	128.00	59.00	129.00
Copper	ppm	17.00	12.00	12.00	11.00	11.00
Zinc	ppm	128.00	84.00	183.00	183.00	183.00

[a] The nutrient analysis of diets C, A and G was determined by Litchfield Analytical Services, Litchfield, MI. The nutrient concentrations for diets A' and G' are based on calculation from diets A and G, and their respective additives.

[b] Data expressed on dry-matter basis.

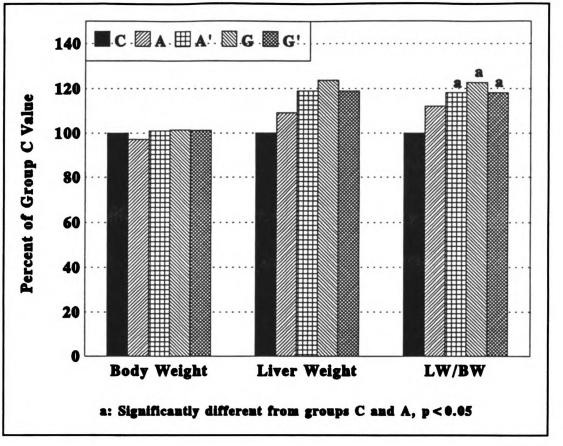

Figure 11. Body and liver weights of F-0 lactating females. F-0 females were provided with treatment diets immediately after parturition and through lactation.

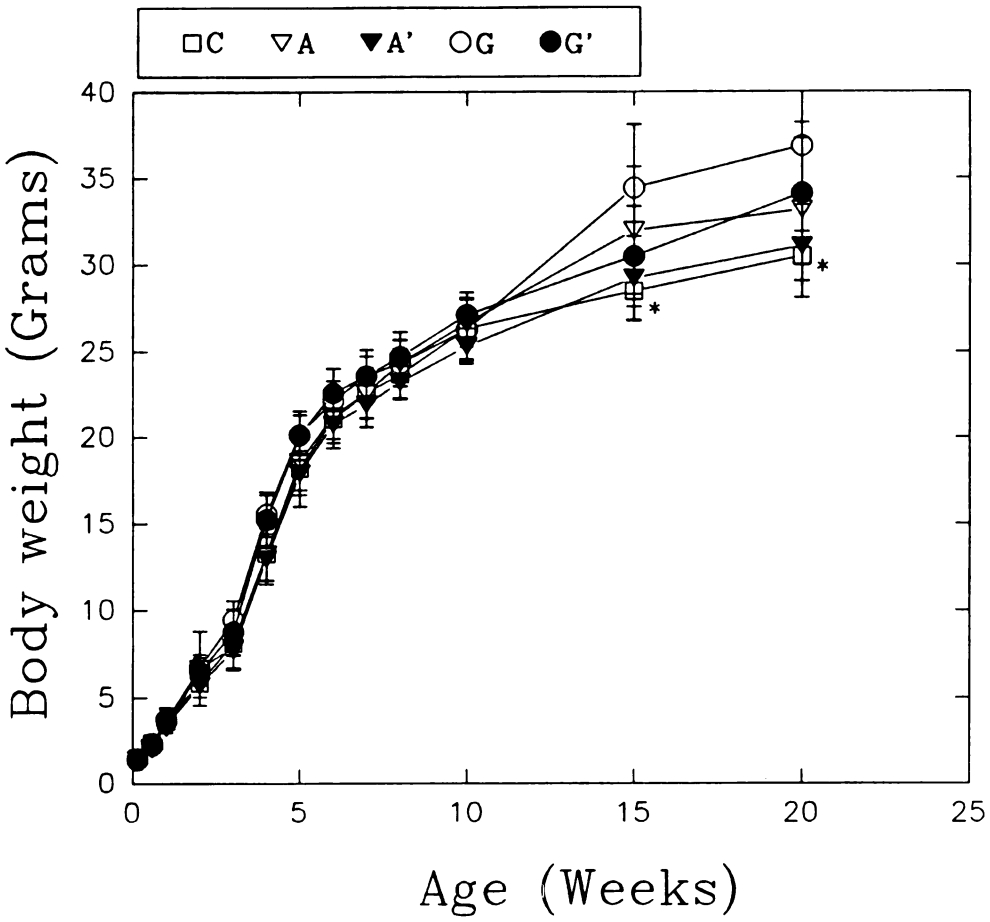

Figure 12. Body weights of F-1 males in Phase III from birth to 20 weeks of age. No difference in body weights among treatment groups was observed before 10 weeks of age. At 15 and 20 weeks of age, mice in Group C showed significantly larger body weights than those in other groups; mice in Groups G and G' were not significantly different in body weights, and were both larger than those of Groups A and A'.

There were no differences in BW between groups A and A', either.

Liver, thymus and testis weights were measured and analyzed in F-1 males at 8 and 11 weeks. Livers in groups G and G' were heavier than those of group A', which were heavier than groups C and A (Figure 13; Appendix 17). At 11 weeks of age, however, LW in group G was heavier than all others. When data were expressed as LW/BW ratio, there were no differences in all 5 groups at 8 and 11 weeks of age. There was no difference in thymus or testis weights among all 5 groups at 8 weeks of age. At 11 weeks of age, the thymus of mice in all 4 fish-diet groups weighed less than those in group C. Mice in group G' had smaller testis than those in groups C, A' and G at 11 weeks.

Liver vitamin (Vit) E concentrations in F-1 males of groups G and G' were lower than those in groups C, A and A' (Figure 14; Appendix 18). Liver Vit A concentrations in groups C and G were similar and were the highest among the treatment groups. Liver Vit A concentrations in groups A and A' were similar and were lower than those in groups C and G, but higher than those in group G'.

To study the reproductive performance of F-1 males, epididymal sperm concentration, motion quality, *in vitro* fertilizing ability and breeding ability were studied. There was no difference in sperm concentration (Figure 15; Appendix 19) or the other parameters measured by the Sperm Analyzer at

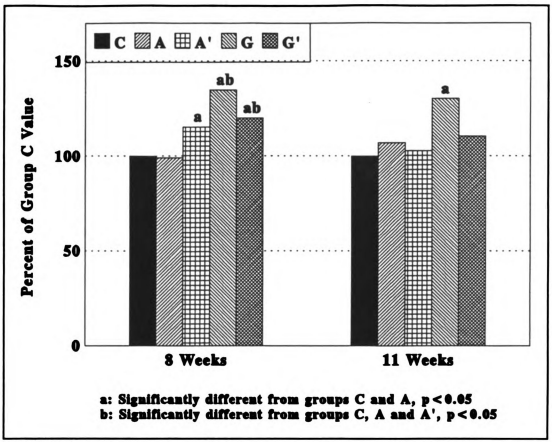

Figure 13. Liver weights of F-1 males at 8 and 11 weeks of age.

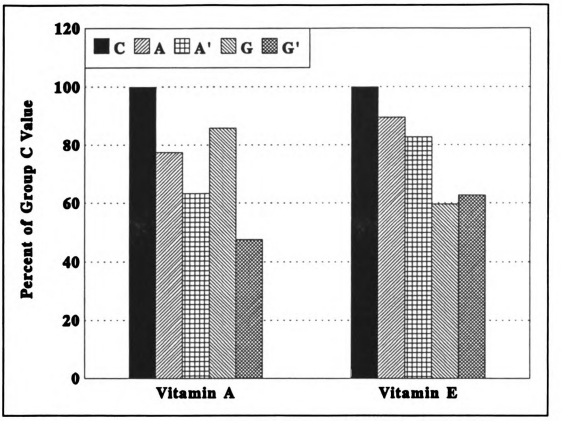

Figure 14. Liver vitamin A and E concentrations in 8-week-old F-1 males.

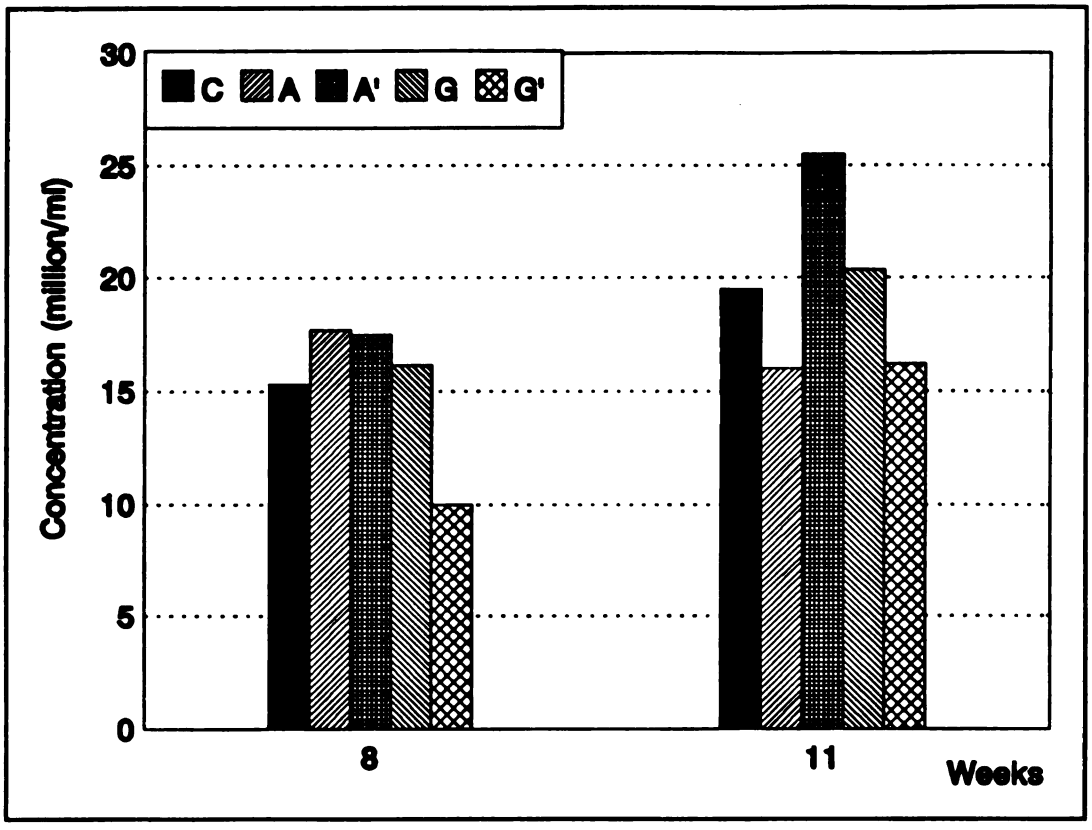

Figure 15. Caudal epididymal sperm concentrations of F-1 males at 8 and 11 weeks of age. Each column represents the mean of 3 samples. Sperm suspension was collected as following: both epididymides were placed in a Falcon Organ Tissue Culture Dish (60 x 15 mm, Becton Dickinson Labware) and poked with a 25 G needle to release the sperm into 1.0 ml BMOC-3 medium. After a 30-min incubation at 37°C, 5% CO_2 in air and 100% humidity, 20 μl of the sperm suspensions were placed on a CellSoft 20μ-chamber and examined under a CellSoft Video-micrographic Computer-Assisted Semen Analyzer (CRYO Resources Inc., New York, NY). A minimum of 100 sperm cells were analyzed for each sample.

both 8 and 11 weeks of age (Appendix 20). Similar to the F-1 males in Phase I, there was no difference in the *in vitro* fertilizing ability of the sperm in all treatment groups (Appendix 21). When mated with non-treated females, only 2 females in group G' were found not pregnant. At weaning, groups G and A' showed smaller litter size than those in group C (Figure 16). There were no significant differences in the gestation index and lactation index.

F-1 Females

There were no differences in body and thymus weights among the 5 groups (Figure 17; Appendix 22). Groups G and G', similar to the F-1 males in this phase of study, had larger LW than those of groups C and A (Figure 17). There was no difference in the superovulation rate or *in vitro* fertilization rate (Appendix 23).

F-2 Males

The BW of F-2 males from birth to 8 weeks of age is shown in Appendix 24. Although the BW of group A' were consistently lower than those of the other groups, no significant difference was observed among groups.

The liver, thymus and testis weights of F-2 males were measured at 5, 6, 7 and 8 weeks of age and are shown in Appendix 25. At weeks 5 and 8, groups G and G' showed larger liver weights than those in group C (Figure 18). However,

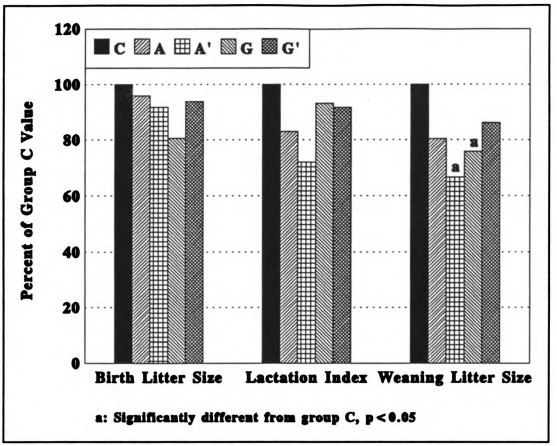

Figure 16. Reproductive performance of 8-week-old F-1 males. Fecundity refers to number of females giving birth/number of females bred. Lactation index refers to percent of F-2 pups survived to the age of 21 days.

Fig

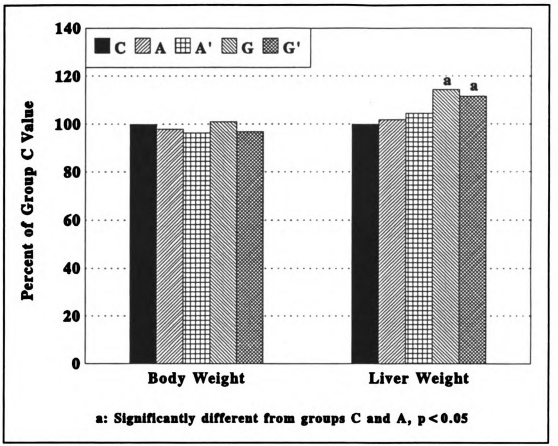

Figure 17. Body and liver weights of 8-week-old F-1 females.

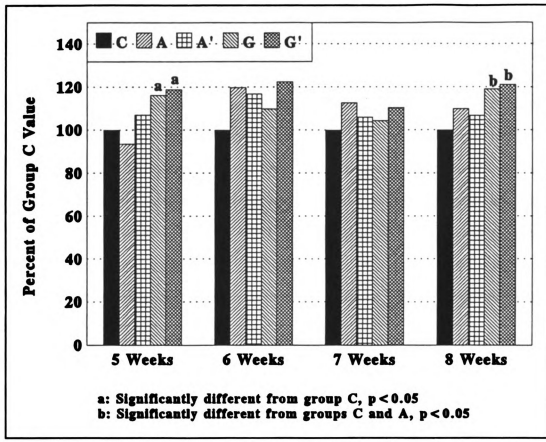

Figure 18. Liver weights of F-2 males at 5, 6, 7, and 8 weeks of age.

when the data were expressed as a LW/BW ratio, no differences were observed at 5 to 8 weeks of age. At week 7, groups A' and G' showed significantly larger thymus weights than groups C and G, but there were no differences at weeks 5, 6 and 8. The testis weights of the mice in groups G and G' were significantly larger than those of groups C and A at 5 weeks of age. There were no differences in testis weight at weeks 6 and 7. Although group G showed significantly larger testis weights than groups A' and G' at 8 weeks of age, no differences were observed when the data were expressed as testis/body weight ratio.

The liver Vit A concentrations of the F-2 males, when expressed as a percentage of group C, were 23.7% in group A, 13.5% in group A', 51.9% in group G ,and 38.8% in group G' (Figure 19; Appendix 26). Liver Vit E concentration was highest in group A (20.33 μg/g of dry LW), followed by 13.02 μg/g in group C, 7.52 μg/g in group A', 5.27 μg/g in group G, and was lowest in group G' (2.79 μg/g).

The assessment of the effects of consuming Great Lakes carp on reproductive performance of the mice was carried on to the second generation (F-2). There was no significant difference in F-2 male sperm concentrations (Figure 20; Appendix 27) or other motion parameters measured at 5, 6, 7, and 8 weeks of age (Appendix 28). *In vitro* fertilizing ability did not differ (Appendix 29). When bred with non-treated females, only 2 females in group C were found not

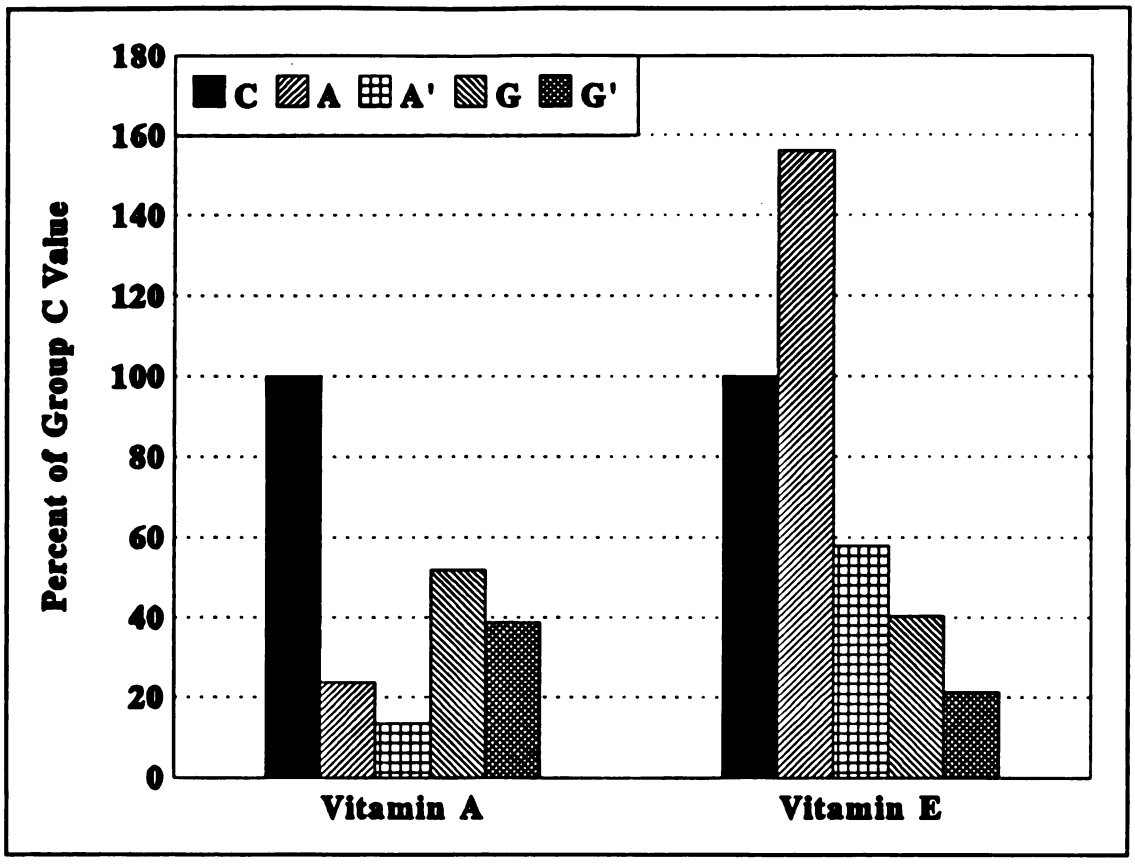

Figure 19. Liver vitamin A and E concentrations on 8-week-old F-2 males.

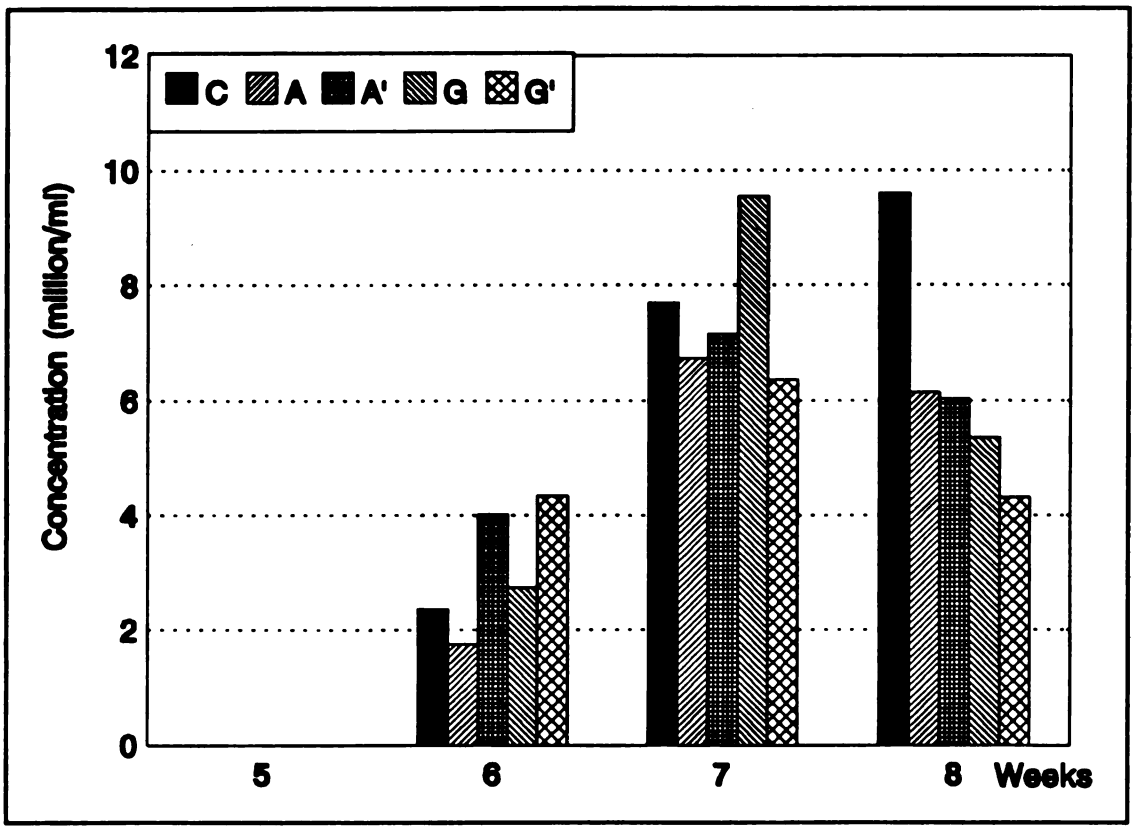

Figure 20. Caudal epididymal sperm concentrations of F-2 males at 5, 6, 7 and 8 weeks of age. Each column represents the mean of 3 samples. Sperm suspension was collected as follows: both epididymides were placed in a Falcon Organ Tissue Culture Dish (60 x 15 mm, Becton Dickinson Labware) and poked with a 25 G needle to release the sperm into 1.0 ml BMOC-3 medium. After a 30-min incubation at 37°C, 5% CO_2 in air and 100% humidity, 20 μl of the sperm suspensions were placed on a CellSoft 20μ-chamber and examined under a CellSoft Video-micrographic Computer-Assisted Semen Analyzer (CRYO Resources Inc., New York, NY). A minimum of 100 sperm cells were analyzed for each sample.

pregnant (Appendix 29). The fecundity of groups A, A', G and G' was 100%. There was no difference in the survival rate at 7 days, although one litter in group A' was found dead at birth. There were no difference in the BW of the pups (F-3) in groups C, A and G, but lower BW were observed in groups A' and G' (Figure 21).

F-2 Females

The body, liver and thymus weights of F-2 females continuously exposed to treatment diets after weaning were measured at 31 to 44 days of age. Similar to the F-1 and F-2 males, group A' had the lowest BW in 5 groups. No differences were found in the BW among the other 4 groups (Figure 22; Appendix 30). The liver weights in group G were higher than those of groups A and A' (Figure 22). However, when the data were expressed as a LW/BW ratio, all the fish-eating groups, A, A', G and G', showed larger LW/BW ratios than group C. There were no differences in the thymus weights among all five groups.

Liver Vit A and E concentrations of the F-2 females are shown in Figure 23 and Appendix 31. Similar to the F-1 males in this phase (III), the total liver Vit A concentrations in groups C (883.7 μg/g of dry LW) and G (576.3 μg/g) were higher than those in groups A (346.3 μg/g) and G' (329.3 μg/g), which were higher than those of group A' (164.3 μg/g). Liver Vit E concentrations in group C (22.56 μg/g) were the highest among

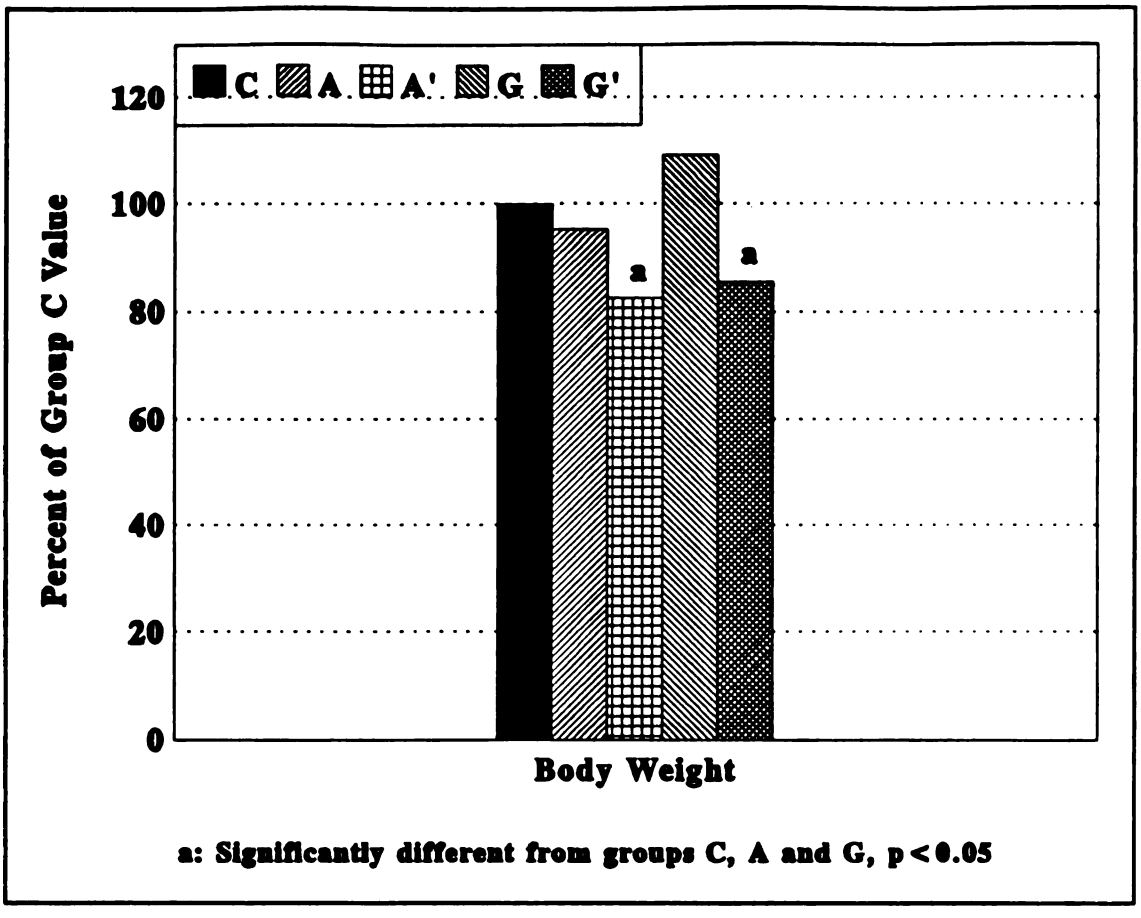

Figure 21. Body weights of 7-day-old F-3 pups.

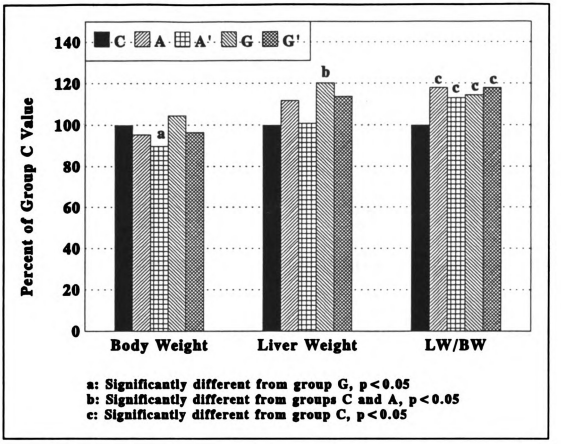

Figure 22. Body and liver weights of F-2 females. F-2 females were between 31 to 44 days old.

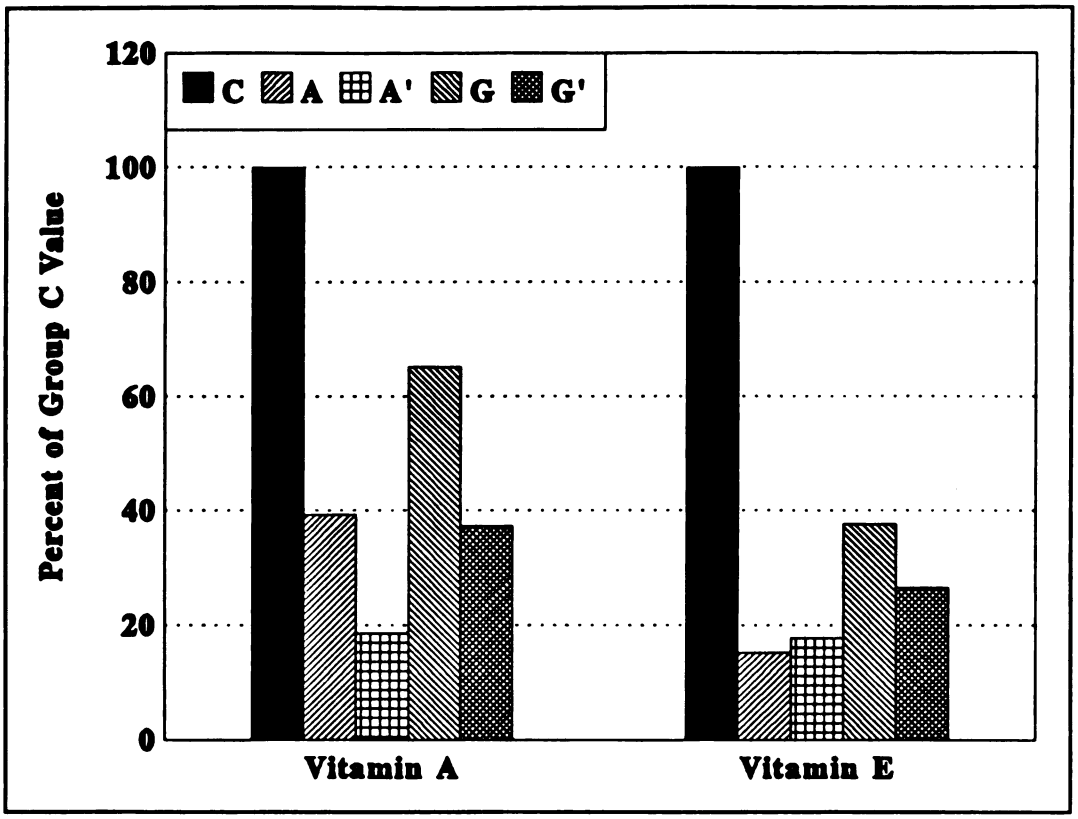

Figure 23. Liver vitamin A and E concentrations in 8-week-old F-2 females.

the 5 groups. Liver Vit E concentrations in group G was 8.49 μg/g of dry LW, which was about one-third the concentration of group C, and was higher than those of groups G', A and A'.

There were no differences in the superovulation rates and the *in vitro* fertilization rates of the F-2 females (Appendix 32).

DISCUSSION

Diets A' and G' were formulated to minimize the differences in nutrient contents between the Arkansas carp and the Great Lakes carp. Because it is difficult to remove excess compounds from the diets, the diets were adjusted by simply adding 100 ppm Zn and 2.8% fish oil to diet A and 0.95% Ca, 0.4% P, 317 ppm Fe, and 70 ppm Mn to diet G (Appendix 13).

The LW in groups C, A and G of the F-0 dams in this phase (Figure 11) were similar to the previous findings of F-0 dams in Phase I (Figure 1). The LW of groups G and G' were the highest of the 5 treatments and lowest in group C. There was no difference in the LW of groups G and G', indicating that the minerals added to diet G had no effect on liver weight. The same situation was also found in groups A and A'.

In the Phase I and III studies, LW were larger in the carp-eating groups than in the lab chow control, indicating that the fish diets used in these two studies could cause liver enlargement. The causative factors within the fish diets are still under investigation. On the other hand, the LW of F-0 dams in group G were consistently larger than those in group A in both Phase I and III, suggesting that some component of the Great Lakes carp, other than in the Arkansas farm-raised carp may be responsible for this additive liver enlargement in the fish-eating mice.

However, in the F-1 males of the Phase I study, there

were no differences in the LW between groups A and G (Figure 4). These findings in the LW between groups A and G were also in agreement with the observations on the F-1 females (Figure 17), F-2 males (Figure 18), and F-2 females (Figure 22) in Phase III. There are two possible explanation for this inconsistency between F-0 and other generations. First, the variation of the BW in F-0 dams among treatment groups was much less than that in other generations. Although data expressed as LW/BW ratio reduced the variations caused by the BW to some extent, it was not perfect enough. When the mice reached 10 weeks of age, body fat accumulations were found in groups C, G and G', but not in groups A and A'. The body fat would decrease the LW/BW ratio by increasing the BW and thus, diminishing the differences between groups A and G. The reason why groups A and A' had less body fat storage is unknown. Second, F-0 dams were exposed to treatment diets right after parturition and through lactation. The different hormone, glucose, and immunoglobulin concentrations, and liver metabolism in gestation and lactation could intense the liver response.

Lower BW in groups A, A' and G' was observed in the F-1 (Figure 12) and F-2 males (Appendix 24) during this phase of the study. In the F-1 males, the BW of groups A and G' were similar and lower than those of group G before 10 weeks of age, indicating that the minerals (Ca, P, Fe and Mn) added to diet G' played some role in decreasing the BW. However, this

finding was only observed before 10 weeks of age. This could also be explained by the body fat deposition. Thus, group G' began showing larger BW levels than group A after 10 weeks of age. The lowest BW were observed consistently in group A'. This may suggest that the extra fish oil supplementation (2.8%) and 100 ppm zinc added in diet A' may even lower the BW further.

Decreasing thymus weight in polyhalogenated aromatic hydrocarbons (PCBs, PBBs, PCDDs, PCDFs, etc.)-treated animals has been reported by others (Faith and Moore, 1977; Vos *et al.*, 1980; Poland and Kuntson, 1982; Vos and Luster, 1989). However, I did not observe any constant change in the thymus weight, nor did I observe any differences in testis weight among the treatments.

Lower liver Vit A and E concentrations in fish-eating groups, similar to those found in the F-0 dam (Figure 2) and F-1 male mice (Figure 6) in Phase I, were also observed in the F-1 males (Figure 14), F-2 males (Figure 19) and F-2 females (Figure 23) during this phase of study. According to the Phase II study, oxidizing agents such as PUFA may be responsible for lowering the liver Vit A and E concentrations. Supplemental nutrients did not interfere with this event. However, the total liver Vit A and E concentrations in group C during this phase were much lower than in Phase I. This alteration may be due to the different mouse strains or other unknown factors.

The results of caudal epididymal sperm concentrations and motion parameters of F-1 and F-2 males in Phase III showed no differences among the 5 groups at all ages measured. There was also no difference in the caudal epididymal sperm fertilization ability *in vitro*. These findings were in agreement with the Phase I study results. Therefore, it may be concluded that eating Great Lakes carp has no effect on caudal epididymal sperm reserves, production, motility, and *in vitro* fertilization ability in mice.

Smaller litter sizes at weaning were observed in groups A' and G in the F-1 males of this phase. The small litter size of group A' at weaning was obviously due to the low survival rate of the F-2 pups (Figure 16). The lower survival index at day 21 in group A' when compared to that in group A suggested that extra fish oil (2.8%) and/or 100 ppm zinc may have some impact on either lactating dams, growing pups or both. Smaller litter size of group G at weaning was mainly due to the smaller litter size at birth. Although there were no significant differences in litter size at birth, group G had about 2 pups less than the control groups (7.9 v.s. 9.8). The consequences of smaller litter size in group G was enhanced by a slightly decreased pup survival rate (83.5% ± 14.5 vs. 89.6 ± 14.6), hence a significant difference in litter size at weaning was shown. The low-birth litter size of F-1 males in group G apparently was not caused by the Great Lakes carp, because a decrease in litter size did not occur in

group G'.

In Phase I, the survival rate of the F-2 pups from both carp-eating groups were lower than that of the lab chow fed controls. The viability of F-1 male pups in group A was only 12%. In this phase of the study, although F-2 pups in groups A and A' still exhibited low survival rates (74.4 ± 16.2 in A and 64.6 ± 28.0 in A' vs. 89.6 ± 14.6 in C), neither was different from the other groups (Appendix 21). The use of a different mouse strain in Phase III could be one of the reasons contributing to the difference. The different survival rate of the group G F-1 male pups showed in Phase I and III could also be explained by the changed mouse strain.

The effects of the fish diets on reproductive performance of female mice was also studied at this phase. In Phase I, F-1 females of group G failed to respond to PMSG/hCG-inducing superovulation at expected time (Figure 9). As discussed previously (pp. 84), the low superovulation rate and numbers of oocyte collected from CBA/CAJ mice may not have been the proper strain for this study. In this phase, there were no differences in F-1 and F-2 females' superovulation rates and *in vitro* fertilization rates. Thus, ingestion of Great Lakes carp had no effect on reproductive performance *in vivo* or *in vitro* for F-1 and F-2 female mice.

SUMMARY

Increased liver weight was observed in the F-0 dams of both carp-eating groups with the effects being more pronounced in the Saginaw Bay carp-fed group. I propose that environmental contaminants within the Saginaw Bay carp, in addition to some indigenous factors in both sources of carp, were responsible for the higher liver weights in the Saginaw Bay carp-fed mice. The increased liver weights, however, were not significant in the F-1 and F-2 generations. The lack of statistically significance in latter generations was probable due to increased variations in both liver weights and body weights. On the other hand, physiological changes such as hormone, immunoglobulin concentrations, and liver metabolism during gestation and lactation may have enhanced the liver response of F-0 dams to the fish diet and the Great Lakes contaminants.

Among all treatment groups, the lowest body weight was observed in the Arkansas carp-fed groups. Adding minerals (Ca, P, Fe, and Mn) to the diet composed of 60% Saginaw Bay carp reduced the body weights of Saginaw Bay carp-fed mice to the weights observed in Arkansas carp-fed mice. Decreased body weight in Arkansas carp-fed mice may have been caused by the high mineral contents in the Arkansas carp diets.

Low hepatic vitamin A and E was observed consistently in both fish-eating groups. Oxidizing agents such as fish oil

may be responsible for these effects. Although decreasing thymus weight in polyhalogenated aromatic hydrocarbon-treated animals was repeatedly reported by others, I did not observe any consistent change on thymus weight, nor did I observe any significant difference in the testis weights among treatments.

From this study, I demonstrated that the diet containing 60% Saginaw Bay carp had no adverse effects on caudal epididymal sperm reserves and motility and *in vitro* fertilizing ability of sperm from the treated mice. No differences in litter size and offspring viability were observed in all three generations. No differences in superovulation rates and *in vitro* fertilizing ability of F-1 and F-2 females suggest that ingestion of Saginaw Bay carp had no any adverse effects on the reproductive performance of female mice *in vitro*.

APPENDICES

APPENDIX 1.

Table 7. Organochlorinated Pesticides and PCBs in Fish Oil.

Pesticide	Concentration (ppm)
PCBs	<IDL
α-BHC	<IDL
ß-BHC	<IDL
Lindane	<IDL
Heptachlor	<IDL
Heptachlor Epoxide	<IDL
Aldrin	<IDL
Technical Chlordane	0.17
p,p'-DDE	0.05
o,p'-DDD	0.02
p,p'-DDD	0.02
o,p'-DDT	<IDL
p,p'-DDT	<IDL
Methoxychlor	<IDL
Dieldrin	0.05
Endrin	<IDL
Malathion	<IDL
Toxaphene	<IDL

Source:
The pesticides and PCBs analysis were performed by Thionville Laboratories, Inc., New Orleans, LA. PCBs and pesticides that were not detected are labeled <IDL. The minimum detection level for PCBs and toxaphene was 0.5 ppm; the minimum detection level for the other pesticides was 0.02 ppm.

APPENDIX 2.

Table 8. Nutrient Requirements of Mice[a].

Minerals	Requirement	Vitamins	Requirement
Macro-minerals (%)			
Calcium	0.4	Vitamin A (IU/kg)	500.0
Magnesium	0.05	Vitamin D (IU/kg)	150.0
Phosphorus	0.4	Vitamin E (IU/kg)	20.0
Potassium	0.2	Vitamin K1 (mg/kg)	3.0
Micro-minerals (mg/kg)		Biotin (mg/kg)	0.2
Chromium	2.0	Choline	600.0
Copper	4.5	Folacin	0.5
Iodine	0.25	Niacin	10.0
Iron	25.0	Pantothenate	10.0
Manganese	45.0	Riboflavin	7.0
Zinc	30.0	Thiamin	5.0
		Vitamin B6	1.0
Crude protein	18.0%	Crude fat	11.0%
Crude fiber	3.0%	Ash	6.5%

Source:
[a] NRC. 1978. Nutrient Requirements of Laboratory Animals.

APPENDIX 3.

Definition of sperm motion parameters:

Concentration:
Number of sperm recovered from epididymides which were poked 30 times and cultured for 30 min within 1 ml of medium.

Percent Motile:
The percentage of sperm that travel more than 20 microns/sec.

Velocity (microns per second):
Average of the distance traveled by motile sperm in microns/sec.

Linearity:
A measure of the straightness of a sperm track on a scale of 0 to 10: 10 indicates a perfectly straight line and 0 indicates a circular track.

A.L.H.(Amplitude of Lateral Head Displacement):
A measure of the displacement of the sperm head from a computer-calculated curval mean of its track in microns/sec. In some species, high A.L.H. appears to be linked with the process of capacitation.

Beat/Cross Frequency (Hz):
The beat/cross frequency (BCF) is reported as the number of beats (or crosses) per second. Every time the sperm cell crosses the computer-calculated curval mean, the computer counts that crossing as one beat. In some studies BCF appears to be reversely related to ALH.

APPENDIX 3. (cont'd)

Table 9. Operating Setting of the CellSoft Videomicrographic Instrument Used for Computer-Assisted Semen Analysis[a]

Parameter[b]	Value
Number of frames per second	30
Number of frames to analyze	15
Maximum velocity (μm/s)	300
Minimum track points for calculation of motility	2
Minimum track points for calculation of velocity	3
Minimum track points for calculation of amplitude of lateral head displacement	7
Minimum velocity for calculation of amplitude of lateral head displacement (μm/s)	20
Minimum linearity for calculation of amplitude of lateral head displacement	3.5

Source:
[a] CellSoft; CRYO Resources, New York, NY.
[b] Ginsburg *et al.* (1990).

APPENDIX 4.

Table 10. Body and liver weights of F-0 lactating mice[a,b].

	Group C	Group A	Group G
Body weight			
Grams	26.16 ± 1.84	26.23 ± 2.33	26.56 ± 2.13
(%)[c]	(100.0)	(100.3)	(101.5)
Liver weight			
Grams	1.84 ± 0.29	2.26[d]± 0.31	2.58[d,e]± 0.24
(%)	(100.0)	(123.0)	(140.9)
Sample size	14	14	16

[a] F-0 females were provided with treatment diets immediately after delivery and through lactation.
[b] Data presented as mean ± standard deviation.
[c] % refers to percent of Group C.
[d] Significantly different from Group C, $p<0.05$.
[e] Significantly different from Group A, $p<0.05$.

APPENDIX 5.

Table 11. Liver vitamin A and E concentrations of lactating F-0 females[a,b,c].

	Group C	Group A	Group G
Retinol	309.0	105.0	91.5
(%)[d]	(100.0)	(34.0)	(29.6)
Retinyl Palmitate	5678.5	2707.5	3104.5
(%)	(100.0)	(47.7)	(54.7)
Total Vitamin A	5987.5	2812.5	3196.0
(%)	(100.0)	(47.0)	(53.4)
Vitamin E	46.81	6.92	9.74
(%)	(100.0)	(14.8)	(20.8)
Sample size	1	1	1

[a] Vitamin A and E analyses were determined by Dr. Stowe's lab., the Animal Health Diagnostic Lab., Nutrition Section, MSU.
[b] F-0 females were provided with treatment diets immediately after delivery and through lactation.
[c] Data expressed as μg/g of dry liver weight.
[d] % refers to percent of Group C.

APPENDIX 6.

Table 12. Body weights of F-1 male mice from birth to 23 weeks of age[a].

Age	Body weight (Grams)		
	Group C	Group A	Group G
Day 1	1.28 ± 0.10	1.28 ± 0.11	1.27 ± 0.10
Day 4	1.98 ± 0.35	1.80 ± 0.36	1.88 ± 0.25
Week 1	3.33 ± 0.62	3.18 ± 0.49	3.15 ± 0.32
Week 2	6.83 ± 0.50	5.92 ± 0.55	6.46 ± 0.55
Week 3	8.73 ± 0.78	7.30 ± 0.96	8.26 ± 0.61
Week 4	13.70 ± 1.22	10.88 ± 1.26	12.98 ± 1.27
Week 5	18.47 ± 1.48	15.72 ± 1.65	17.38 ± 1.14
Week 6	21.00 ± 1.41	18.01 ± 1.46	19.48 ± 1.47
Week 7	22.76 ± 1.63	20.02 ± 1.20	21.52 ± 1.32
Week 8	24.34 ± 2.13	21.60 ± 1.36	23.22 ± 1.09
Week 10	26.03 ± 1.84	23.71 ± 1.44	25.98 ± 1.62
Week 14	31.24 ± 0.35	26.20 ± 1.70	33.62 ± 2.13
Week 15	30.23 ± 2.76	27.58 ± 1.83	30.51 ± 0.78
Week 21	36.54 ± 0.77	29.33 ± 2.48	32.28 ± 1.34
Week 23	39.76 ± 1.26	31.43 ± 0.27	31.44 ± 2.04

[a] Data presented as mean ± standard deviation.

APPENDIX 7.

Table 13. Liver and testis weights of F-1 males[a].

	Group C	Group A	Group G
7 weeks of age			
Liver weight			
Grams	1.42 ± 0.22	1.46 ± 0.10	1.50 ± 0.10
(%)[b]	(100.0)	(102.8)	(105.6)
Body weight			
Grams	23.14 ± 0.99	19.65 ± 1.60	19.92 ± 1.87
(%)	(100.0)	(84.9)	(86.1)
Liver/body weight ratio	0.061	0.074[c]	0.075[c]
(%)	(100.0)	(121.3)	(123.0)
Testis weight			
Grams	0.12 ± 0.01	0.11 ± 0.01	0.11 ± 0.01
(%)	(100.0)	(91.7)	(91.7)
Sample size	3	3	3
8 weeks of age			
Liver weight			
Grams	1.52 ± 0.14	1.49 ± 0.08	1.63 ± 0.08
(%)	(100.0)	(98.0)	(107.2)
Body weight			
Grams	24.89 ± 2.00	22.34 ± 0.26	24.25 ± 0.62
(%)	(100.0)	(89.8)	(97.4)
Liver/body weight ratio	0.061	0.067[c]	0.067[c]
(%)	(100.0)	(109.8)	(109.8)
Testis weight			
Grams	0.12 ± 0.01	0.12 ± 0.01	0.12 ± 0.01
(%)	(100.0)	(100.0)	(100.0)
Sample size	3	3	3

Table 13 (cont'd)

15 weeks of age

Liver weight Grams (%)	1.81 ± 0.15 (100.0)	1.47[d] ± 0.16 (81.2)	1.86 ± 0.11 (102.8)
Body weight Grams (%)	34.60 ± 2.13 (100.0)	26.89[d] ± 1.03 (77.7)	31.28 ± 1.86 (90.4)
Liver/body weight ratio (%)	0.052 (100.0)	0.054 (103.8)	0.059 (113.5)
Testis weight Grams (%)	0.13 ± 0.01 (100.0)	0.12 ± 0.02 (92.3)	0.13 ± 0.00 (100.0)
Sample size	3	3	3

[a] Data presented as mean ± standard deviation.
[b] % refers to percent of Group C.
[c] Significantly different from Group C, $p<0.05$.
[d] Significantly different from Groups C and G, $p<0.05$.

APPENDIX 8.

Table 14. Liver vitamin A and E concentrations in F-1 males[a,b].

	Group C	Group A	Group G
19 weeks of age			
Retinol	147	86	109
(%)[c]	(100.0)	(58.5)	(74.1)
Retinyl Palmitate	7754	1906	2979
(%)	(100.0)	(24.6)	(38.4)
Total Vitamin A	7901	1992	3088
(%)	(100.0)	(25.2)	(39.1)
Vitamin E	77.79	9.26	12.96
(%)	(100.0)	(11.9)	(16.7)
Sample size	1	1	1
32 weeks of age			
Retinol	273	182	166
(%)	(100.0)	(66.7)	(60.8)
Retinyl Palmitate	3781	1432	2004
(%)	(100.0)	(37.9)	(53.0)
Total Vitamin A	4054	1614	2170
(%)	(100.0)	(39.8)	(53.5)
Vitamin E	36.74	6.90	13.77
(%)	(100.0)	(18.8)	(37.5)
Sample size	1	1	1

[a] Vitamin A and E analyses were determined by Dr. Stowe's lab., the Animal Health Diagnostic Lab., Nutrition Section, MSU.
[b] Data expressed as μg/g of dry liver weight.
[c] % refers to percent of Group C.

APPENDIX 9.

Table 15. Caudal epididymal sperm concentrations of F-1 males at 6, 7, 8, 15, 23, 32, and 34 weeks of age[a,b].

	Sperm concentration (million/ml)		
Age (weeks)	Group C	Group A	Group G
6	0.97 ± 0.15	0.63 ± 0.15	1.70 ± 0.17
7	6.47 ± 3.18	3.63 ± 0.93	3.47 ± 1.50
8	12.44 ± 0.95	10.59 ± 0.87	13.76 ± 5.43
15	11.42 ± 3.78	15.93 ± 1.35	13.63 ± 2.18
23	11.74 ± 0.80	15.00 ± 8.48	7.06 ± 0.46
32	8.61 ± 2.17	15.41 ± 1.50	15.08 ± 0.90
34	10.67 ± 2.26	10.26 ± 4.28	8.70 ± 0.57

[a] Data presented as mean ± standard deviation.

[b] Sperm suspension was collected as followings: both of the epididymides were placed in a Falcon Organ Tissue Culture Dish (60 x 15 mm, Becton Dickinson Labware) and poked with a 25 G needle to release the sperm into 1.0 ml BMOC-3 medium. After a 30-min incubation at 37°C, 5% CO_2 in air and 100% humidity, 20 μl of the sperm suspensions were placed on a CellSoft 20μ-chamber and examined under a CellSoft Video-micrographic Computer-Assisted Semen Analyzer (CRYO Resources Inc., New York, NY). A minimum of 100 sperm cells were analyzed for each sample.

APPENDIX 10.

Table 16. Epididymal sperm motion quality of F-1 males[a,b].

Parameters[c]	Group C	Group A	Group G
6 weeks of age			
% motile sperm	74.07 ± 3.84	50.90 ± 27.27	78.70 ± 5.48
Velocity(μm/s)	172.07 ± 24.14	98.60 ± 11.32	230.16 ± 69.71
Linearity	6.65 ± 2.07	4.30 ± 1.95	5.22 ± 1.11
A.L.H.[d] (μm/s)	4.44 ± 3.37	5.77 ± 6.36	1.89 ± 1.06
B./C. F.[e] (Hz)	12.81 ± 2.21	12.58 ± 1.76	16.07 ± 8.13
Sample size	3	3	3
7 weeks of age			
% motile sperm	74.30 ± 6.32	60.50 ± 6.33	45.47 ± 5.82
Velocity(μm/s)	170.13 ± 8.06	160.27 ± 16.79	143.56 ± 44.05
Linearity	4.55 ± 0.25	4.48 ± 0.60	4.33 ± 1.91
A.L.H. (μm/s)	5.08 ± 2.31	3.56 ± 0.84	2.71 ± 0.39
B./C. F. (Hz)	11.59 ± 1.05	13.13 ± 2.10	10.65 ± 1.34
Sample size	3	3	3
15 weeks of age			
% motile sperm	81.82 ± 4.35	81.68 ± 3.94	82.15 ± 5.69
Velocity(μm/s)	220.88 ± 20.16	228.17 ± 24.43	226.60 ± 20.13
Linearity	4.78 ± 0.91	4.45 ± 0.32	4.83 ± 0.50
A.L.H. (μm/s)	7.72 ± 1.69	10.24 ± 3.51	9.52 ± 0.33
B./C. F. (Hz)	12.74 ± 0.81	12.43 ± 1.23	9.46 ± 2.72
Sample size	3	3	3

[a] For sperm motion quality analysis, 20 μl of the sperm suspension were placed on a CellSoft 20 μ chamber and examined under a CellSoft Videomicrographic Computer-Assisted Semen Analyzer (CRYO Resources Inc., New York, NY). A minimum of 100 sperm cells were analyzed for each sample. Sperm suspension was collected from epididymides and had been incubated in BMOC-3 medium for one half-hour at 37°C, 5% CO_2 in air, 100% humidity.

[b] Data presented as mean ± standard deviation.
No statistical significance was observed among the groups, $p > 0.05$.

[c] See Appendix 3 for definition of parameters.

[d] A.L.H. represents amplitude of lateral head displacement.

[e] B./C. F. represents beat/cross frequency.

APPENDIX 11.

Table 17. Reproductive performance of F-1 males[a,b].

Parameters	Group C	Group A	Group G
In vitro fertilizing ability of epididymal sperm[c]			
No. of oocyte tested	22.5 ± 2.1	33.3 ± 11.4	27.0 ± 7.8
IVF[d] rate (%)	86.4 ± 7.5	73.6 ± 9.65	83.6 ± 14.57
Sample size	3	3	3
Breeding ability[e]			
Fecundity[f]	7/7	7/7	7/7
Litter size	8.0 ± 1.0	6.0 ± 2.1	7.3 ± 2.8
Viability[g]	94.3 ± 10.2	12.2[h] ± 32.4	44.6[h] ± 43.2
Sample size	7	7	7

[a] Data presented as mean ± standard deviation.
[b] F-1 males were 52 days old (7.5 weeks).
[c] To determine the *in vitro* fertilizing ability, sperm was used to inseminate oocytes collected by superovulation from previously non-treated females. The sperm suspension was collected from treated male epididymides and had been incubated in BMOC-3 medium for one half-hour at 37°C, 5% CO_2 in air, 100% humidity. Oocytes were inseminated with 50 μl of sperm suspension (1-3 x 10^7) and were co-incubated at 37°C, 5% CO_2 in air, 100% humidity for 24 hours before examination of fertilization.
[d] IVF refers to *in vitro* fertilization.
[e] For breeding, 7 F-1 males were each paired with a previously non-treated mature female. The females began to receive the same treatment diets as the males when they were housed together for 5 days. These females were then housed individually and continuously provided with treatment diets through gestation and lactation.
[f] Fecundity refers to number of females giving birth/number of females bred.
[g] Viability refers to percent of F-2 mice that survived to the age of 7 days.
[h] Significantly different from Group C, $p<0.05$.

APPENDIX 12.

Table 18. Reproductive performance of F-1 females.

Parameters	Group C	Group A	Group G
In vitro fertilizing ability of oocytes[a]			
Superovulation rate[b]	7/8	4/6	0/7
% females ovulated	87	67[f]	0[f,g]
No. of oocyte ovulated	9.6 ± 7.9	9.8 ± 3.3	0
% oocytes fertilized[c]	62.9 ± 19.1	59.8 ± 22.8	---
Sample size	8	6	7
Breeding ability[d]			
Fecundity[e]	7/7	3/6	4/7
% of Group C	100	50.0[f]	57.1[f]
Litter size[c]	7.0 ± 2.6	6.3 ± 1.5	6.0 ± 0.82
Sample size	7	6	7

[a] To determine the oocyte *in vitro* fertilizing ability, oocytes were inseminated with sperm from previously non-treated males. Oocytes were collected from PMSG/hCG superovulated treated females. The ages were 5 and 10 weeks old. The sperm suspension was collected from mature male epididymides and had been incubated in BMOC-3 medium for one half-hour at 37°C, 5% CO_2 in air, 100% humidity. Oocytes were inseminated with 50μl of sperm suspension (1-3 x 10^7) and were co-incubated at 37°C, 5% CO_2 in air, 100% humidity for 24 hours before examination of fertilization.

[b] Superovulation rate refers to number of females ovulated/number of females superovulated with PMSG/hCG.

[c] Data presented as mean ± standard deviation.

[d] For breeding, F-1 females were paired each with a previously non-treated mature male. The males have never received any treatment diet even during the 5-day breeding period.

[e] Fecundity refers to number of females carrying fetus 10 days after breeding period/number of females bred.

[f] Significantly different from Group C, $p<0.05$.

[g] Significantly different from Groups C and A, $p<0.05$.

APPENDIX 13.

Table 19. Liver vitamin A and E concentrations of F-1 males after high dietary vitamin E Supplementation[a,b,c].

	Group C	Group A+	Group G+
10 days supplement			
Retinol	309.0	629.0	419.0
(%)[d]	(100.0)	(203.6)	(135.6)
Retinyl Palmitate	6133.0	3785.0	3382.0
(%)	(100.0)	(61.7)	(55.1)
Total Vitamin A	6442.0	4414.0	3801.0
(%)	(100.0)	(68.5)	(59.0)
Vitamin E	19.32	76.19	112.89
(%)	(100.0)	(394.4)	(584.3)
30 days supplement			
Retinol	130	544	162
(%)	(100.0)	(418.5)	(124.6)
Retinyl Palmitate	3797	5522	3760
(%)	(100.0)	(145.4)	(99.0)
Total Vitamin A	3927	6066	3930
(%)	(100.0)	(154.5)	(100.1)
Vitamin E	40.79	97.72	129.45
(%)	(100.0)	(239.6)	(313.4)
156 days supplement			
Retinol	287	585	464
(%)	(100.0)	(203.8)	(161.7)
Retinyl Palmitate	2700	4855	3924
(%)	(100.0)	(179.8)	(145.3)
Total Vitamin A	2987	5440	4388
(%)	(100.0)	(182.1)	(146.9)
Vitamin E	30.57	77.30	98.30
(%)	(100.0)	(252.9)	(321.6)

[a] Vitamin A and E analyses were determined by Dr. Stowe's lab., the Animal Health Diagnostic Lab., Nutrition Section, MSU.
[b] Mice were fed high Vit E diets (A+ or G+) beginning at 81 days of age. From birth to 81 days of age, mice were fed treatment diets A or G, respective.
[c] Data expressed as μg/g of dry liver weight.
[c] % refers to percent of Group C.

APPENDIX 14.

Table 20. Body and liver weights of F-1 males after high dietary vitamin E Supplementation[a].

	Group C	Group A+	Group G+
10 days supplement			
Body weight			
Grams	37.40	27.43	30.62
(%)[b]	(100.0)	(73.34)	(81.87)
Liver weight			
Grams	1.837	1.429	1.540
(%)	(100.0)	(77.79)	(83.83)
Liver/body weight ratio	0.049	0.052	0.050
(%)	(100.0)	(106.12)	(102.04)
30 days supplement			
Body weight			
Grams	38.32	26.96	33.72
(%)	(100.0)	(70.35)	(88.00)
Liver weight			
Grams	1.854	1.156	1.856
(%)	(100.0)	(62.35)	(100.11)
Liver/body weight ratio	0.048	0.043	0.055
(%)	(100.0)	(89.58)	(114.58)
156 days supplement			
Body weight	42.61	36.90	37.76
Grams	(100.0)	(86.60)	(88.62)
(%)			
Liver weight	2.181	1.723	1.949
Grams	(100.0)	(79.00)	(89.36)
(%)			
Liver/body weight ratio	0.051	0.047	0.052
(%)	(100.0)	(92.16)	(101.96)
Sample size	1	1	1

[a] Mice were fed high Vit E diets (A+ or G+) beginning at 81 days of age. From birth to 81 days of age, mice were fed treatment diets A or G, respective.

[b] % refers to percent of Group C.

APPENDIX 15.

Table 21. Body and liver weights of F-0 lactating females[a,b].

	Group C	Group A	Group A'	Group G	Group G'
Liver weight					
Grams	1.64 ± 0.30	1.79 ± 0.25	1.95 ± 0.19	2.03 ± 0.26	1.95 ± 0.27
(%)[c]	(100.0)	(109.1)	(118.9)	(123.8)	(118.9)
Body weight					
Grams	24.75 ± 1.97	24.06 ± 1.30	25.00 ± 1.38	25.12 ± 0.60	25.08 ± 2.26
(%)	(100.0)	(97.2)	(101.0)	(101.5)	(101.3)
Liver weight /Body weight Ratio	0.066 ± 0.01	0.074 ± 0.01	0.078[d] ± 0.00	0.081[d] ± 0.01	0.078[d] ± 0.00
(%)	(100.0)	(112.1)	(118.2)	(122.7)	(118.2)
Sample size	5	6	6	7	7

[a] F-0 females were provided with treatment diets immediately after delivery and through lactation.
[b] Data presented as mean ± standard deviation.
[c] % refers to percent of Group C.
[d] Significantly different from Groups C and A, $p<0.05$.

APPENDIX 16.

Table 22. Body weights of F-1 male mice from birth to 20 weeks of age[a].

Age	Body weight (Grams)				
	Group C	Group A	Group A'	Group G	Group G'
Day 1	1.37 ± 0.09	1.39 ± 0.10	1.52 ± 0.12	1.47 ± 0.13	1.39 ± 0.09
Day 4	2.20 ± 0.23	2.12 ± 0.23	2.38 ± 0.37	2.35 ± 0.49	2.00 ± 0.29
Week 1	3.65 ± 0.43	3.41 ± 0.46	3.64 ± 0.69	3.74 ± 0.69	3.27 ± 0.56
Week 2	6.74 ± 0.75	5.71 ± 0.42	5.85 ± 0.81	6.51 ± 0.80	6.68 ± 2.13
Week 3	9.47 ± 1.07	7.76 ± 1.05	8.14 ± 0.73	8.77 ± 1.29	7.91 ± 1.32
Week 4	15.55 ± 1.13	12.98 ± 1.28	13.27 ± 1.76	15.23 ± 1.61	13.24 ± 1.49
Week 5	20.15 ± 1.16	17.93 ± 1.28	18.21 ± 2.21	20.12 ± 1.42	18.57 ± 1.63
Week 6	22.14 ± 1.16	20.75 ± 1.40	21.05 ± 1.37	22.59 ± 1.44	21.22 ± 1.32
Week 7	23.57 ± 1.16	21.92 ± 1.34	22.58 ± 1.50	23.57 ± 1.52	22.59 ± 1.48
Week 8	24.32 ± 1.33	23.25 ± 0.98	23.69 ± 1.48	24.71 ± 1.40	24.33 ± 1.37
Week 10	26.27 ± 1.83	25.31 ± 1.02	26.34 ± 1.77	27.11 ± 1.28	26.62 ± 1.39
Week 15	34.42 ± 3.67	29.23 ± 2.42	28.48 ± 1.71	30.48 ± 2.89	31.98 ± 3.68
Week 20	36.86 ± 3.44	31.05 ± 2.96	30.47 ± 1.42	34.10 ± 4.12	33.18 ± 4.12

[a] Data presented as mean ± standard deviation.

APPENDIX 17.

Table 23. Liver, thymus and testis weights of F-1 males[a].

	Group C	Group A	Group A'	Group G	Group G'
8 weeks of age					
Liver weight					
Grams	1.29 ± 0.14	1.28 ± 0.13	1.49[c] ± 0.22	1.74[c,d] ± 0.15	1.55[c,d] ± 0.09
(%)[b]	(100.0)	(99.2)	(115.5)	(134.9)	(120.2)
Liver/body weight ratio	0.053	0.071	0.064	0.055	0.063
(%)	(100.0)	(140.0)	(120.8)	(103.8)	(118.9)
Thymus weight					
Milligrams	38.1 ± 0.8	32.9 ± 2.5	36.4 ± 4.6	35.4 ± 4.5	37.9 ± 9.2
(%)	(100.0)	(86.4)	(95.5)	(92.9)	(99.5)
Testis weight					
Grams	0.19 ± 0.01	0.20 ± 0.03	0.19 ± 0.01	0.21 ± 0.01	0.17 ± 0.04
(%)	(100.0)	(105.3)	(100.0)	(110.5)	(89.5)
Sample size	3	3	3	3	3
11 weeks of age					
Liver weight					
Grams	1.42 ± 0.33	1.52 ± 0.29	1.46 ± 0.32	1.85[c] ± 0.29	1.57 ± 0.14
(%)	(100.0)	(107.0)	(102.8)	(130.3)	(110.6)
Liver/body weight ratio	0.051	0.059	0.055	0.066	0.060
(%)	(100.0)	(115.7)	(107.8)	(129.1)	(117.6)
Thymus weight					
Milligrams	41.0 ± 7.4	25.7[e] ± 5.0	28.9[e] ± 6.0	29.3[e] ± 9.3	28.5[e] ± 5.0
(%)	(100.0)	(62.7)	(70.5)	(71.5)	(69.5)
Testis weight					
Grams	0.22 ± 0.02	0.20 ± 0.04	0.22 ± 0.03	0.23 ± 0.02	0.17[f] ± 0.05
(%)	(100.0)	(90.9)	(100.0)	(104.5)	(77.3)
Sample size	3	3	3	3	3

[a] Data presented as mean ± standard deviation.
[b] % refers to percent of Group C.
[c] Significantly different from Groups C and A, $p<0.05$.
[d] Significantly different from Groups C, A and A', $p<0.05$.
[e] Significantly different from Group C, $p<0.05$.
[f] Significantly different from Groups C, A' and G, $p<0.05$.

APPENDIX 18.

Table 24. Liver vitamin A and E concentrations in F-1 males[a,b,c].

	Group C	Group A	Group A'	Group G	Group G'
Retinol (%)[d]	247 (100.0)	224.5 (90.9)	252.5 (102.2)	333.5 (135.0)	177 (71.7)
Retinyl Palmitate (%)	941 (100.0)	695.5 (73.9)	500 (53.1)	688 (73.1)	388 (41.2)
Total Vitamin A (%)	1188 (100.0)	920 (77.4)	752.5 (63.3)	1021.5 (86.0)	565 (47.6)
Vitamin E (%)	15.91 (100.0)	14.25 (89.6)	13.18 (82.8)	9.48 (59.6)	9.97 (62.7)
Sample size	2	2	2	2	2

[a] Vitamin A and E analyses were determined by Dr. Stowe's lab., the Animal Health Diagnostic Lab., Nutrition Section, MSU.
[b] F-1 males were 8 weeks of age.
[c] Data expressed as μg/g of dry liver weight.
[d] % refers to percent of Group C.

APPENDIX 19.

Table 25. Caudal epididymal sperm concentrations of F-1 males at 8 and 11 weeks of age[a,b].

	Sperm concentration (million/ml)				
Age	Group C	Group A	Group A'	Group G	Group G'
8 weeks	15.32 ± 0.15	12.70 ± 5.29	17.51 ± 2.58	16.16 ± 5.20	9.90 ± 4.15
Sample size	5	5	5	5	5
11 weeks	19.49 ±6.06	16.20 ± 8.12	25.49 ± 4.88	20.34 ± 5.97	16.21 ±12.53
Sample size	8	8	8	8	8

[a] Data presented as mean ± standard deviation.

[b] Sperm suspension was collected as followings: both of the epididymides were placed in a Falcon Organ Tissue Culture Dish (60 x 15 mm, Becton Dickinson Labware) and poked with a 25 G needle to release the sperm into 1.0 ml BMOC-3 medium. After a 30-min incubation at 37°C, 5% CO_2 in air and 100% humidity, 20 μl of the sperm suspensions were placed on a CellSoft 20μ-chamber and examined under a CellSoft Video-micrographic Computer-Assisted Semen Analyzer (CRYO Resources Inc., New York, NY). A minimum of 100 sperm cells were analyzed for each sample.

APPENDIX 20.

Table 26. Epididymal sperm motion quality of F-1 males[a,b].

Parameters[c]	Group C	Group A	Group A'	Group G	Group G'
8 weeks of age					
% motile sperm	64.4 ± 11.9	62.8 ± 15.5	71.9 ± 11.5	61.5 ± 17.1	59.4 ± 14.7
Velocity	173.8 ± 33.1	209.3 ± 17.6	211.9 ± 24.3	191.4 ± 9.7	178.2 ± 30.0
Linearity	4.4 ± 0.6	4.6 ± 0.5	4.3 ± 0.4	4.4 ± 0.6	4.4 ± 0.3
A.L.H.[d]	7.4 ± 2.4	6.8 ± 2.1	7.7 ± 1.0	7.9 ± 1.7	7.8 ± 4.5
B./C. F.[e]	11.9 ± 2.0	14.0 ± 2.0	11.7 ± 0.5	11.2 ± 3.6	10.7 ± 1.5
Sample size	3	3	3	3	3
11 weeks of age					
% motile sperm	68.3 ± 17.9	65.1 ± 8.3	72.8 ± 13.1	71.8 ± 6.5	56.6 ± 19.0
Velocity	193.4 ± 29.4	184.0 ± 33.1	205.4 ± 41.0	203.7 ± 18.8	184.2 ± 27.6
Linearity	4.4 ± 0.3	4.1 ± 0.8	4.4 ± 0.5	4.3 ± 0.4	4.9 ± 0.7
A.L.H.	8.3 ± 2.5	7.4 ± 3.1	10.0 ± 5.2	8.0 ± 2.3	6.0 ± 3.5
B./C. F.	12.7 ± 2.3	12.4 ± 2.5	11.7 ± 1.3	12.8 ± 2.4	12.4 ± 2.4
Sample size	3	3	3	3	3

[a] For sperm motion quality analysis, 20 μl of the sperm suspension were placed on a CellSoft 20 μ chamber and examined under a CellSoft Video-micrographic Computer-Assisted Semen Analyzer (CRYO Resources Inc., New York, NY). A minimum of 100 sperm cells were analyzed for each sample. Sperm suspension was collected from treated male epididymides and had been incubated in BMOC-3 medium for one half-hour at 37°C, 5% CO_2 in air, 100% humidity.

[b] Data presented as mean ± standard deviation.
No statistical significance was observed among the groups, $p > 0.05$.

[c] See Appendix 3 for definition of parameters.

[d] A.L.H. represents amplitude of lateral head displacement.

[e] B./C. F. represents beat/cross frequency.

APPENDIX 21.

Table 27. Reproductive performance of F-1 males[a,b].

Parameters	Group C	Group A	Group A'	Group G	Group G'
In vitro fertilizing ability of epididymal sperm[c]					
No. of oocyte tested	28.5 ± 4.3	26.3 ± 5.7	35.0 ± 7.0	25.6 ± 3.3	38.8 ± 5.9
IVF[d] rate (%)	82.0 ± 14.0	73.1 ± 28.4	85.6 ± 6.8	95.7 ± 5.6	59.6 ± 35.0
Sample size	3	3	3	3	3
Breeding ability[e]					
Fecundity[f]	8/8	8/8	8/8	8/8	6/8
Litter size	9.8 ± 1.7	9.4 ± 1.2	9.0 ± 2.1	7.9 ± 1.8	9.2 ± 1.8
Gestation index[g]	89.6 ± 14.6	90.2 ± 14.4	75.3 ± 24.8	84.6 ± 13.9	82.2 ± 17.9
Lactation index[h]	89.6 ± 14.6	74.4 ± 16.2	64.6 ± 28.0	83.5 ± 14.5	82.2 ± 17.9
Litter size at weaning	8.7 ± 0.2	7.0 ± 0.2	5.8[i] ± 0.6	6.6[i] ± 0.3	7.5 ± 0.3
Sample size	8	8	8	8	8

[a] Data presented as mean ± standard deviation.
[b] F-1 males were 56 days old (8 weeks).
[c] To determine the sperm *in vitro* fertilizing ability, sperm was used to inseminate oocytes which were collected from PMSG/hCG superovulated previously non-treated females. The sperm suspension was collected from treated male epididymides and had been incubated in BMOC-3 medium for one half-hour at 37°C, 5% CO_2 in air, 100% humidity. Oocytes were inseminated with 50 μl of sperm suspension (1-3 x 10^7) and were co-incubated at 37°C, 5% CO_2 in air, 100% humidity for 24 hours before examination of fertilization.
[d] IVF refers to *in vitro* fertilization.
[e] For breeding, 7 F-1 males were paired each with a previously non-treated mature female. The females began to receive the same treatment diets as the males when they were housed together for 5 days. These females were then housed individually and continuously provided with treatment diets through gestation and lactation.
[f] Fecundity refers to number of females giving birth/number of females bred.
[g] Gestation index refers to percent of F-2 survived to the age of 4 days.
[h] Lactation index refers to percent of F-2 survived to the age of 21 days.
[i] Significantly different from Group C, $p<0.05$.

APPENDIX 22.

Table 28. Body, liver and thymus weights of F-1 females[a,b].

Parameters	Group C	Group A	Group A'	Group G	Group G'
Body weight					
Grams	19.5 ± 1.2	19.1 ± 0.8	18.8 ± 1.8	19.7 ± 1.1	18.9 ± 1.4
(%)[c]	(100.0)	(97.9)	(96.4)	(101.0)	(96.9)
Liver weight					
Grams	1.11 ± 0.12	1.13 ± 0.10	1.16 ± 0.14	1.27[d] ± 0.08	1.24[d] ± 0.15
(%)	(100.0)	(101.8)	(104.5)	(114.4)	(111.7)
Thymus weight					
Milligrams	40.7 ± 12.3	46.9 ± 11.0	43.6 ± 9.4	42.5 ± 12.5	44.5 ± 13.1
(%)	(100.0)	(115.2)	(107.1)	(104.4)	(109.3)
Sample size	15	15	15	15	15

[a] F-1 females were 8 weeks ± 2 days.
[b] Data presented as mean ± standard deviation.
[c] % refers to percent of Group C.
[d] Significantly different from Groups C and A, $p<0.05$.

APPENDIX 23.

Table 29. *In vitro* fertilizing ability of F-1 females[a].

Parameters	Group C	Group A	Group A'	Group G	Group G'
Superovulation rate[b]	14/15	14/15	14/15	15/15	10/15
No. of oocyte ovulated[c]	43.2 ± 13.6	39.0 ± 17.2	25.3 ± 17.5	34.6 ± 17.5	33.4 ± 24.7
% oocytes fertilized[c]	92.8 ± 12.4	89.6 ± 6.9	87.0 ± 10.0	83.2 ± 18.3	83.4 ± 20.3
Sample size	3	3	3	3	3

[a] To determine the oocyte *in vitro* fertilizing ability, oocytes were inseminated with sperm from previously non-treated males. Oocytes were collected from PMSG/hCG superovulated treated females. The ages were between 31 to 44 days old. The sperm suspension was collected from mature male epididymides and had been incubated in BMOC-3 medium for one half-hour at 37°C, 5% CO_2 in air, 100% humidity.
Oocytes were inseminated with 50 μl of sperm suspension (1-3 x 10^7) and were co-incubated at 37°C, 5% CO_2 in air, 100% humidity for 24 hours before examination for fertilization.

[b] Superovulation rate refers to number of females ovulated/number of females superovulated with PMSG/hCG.

[c] Data presented as mean ± standard deviation.

APPENDIX 24.

Table 30. Body weights of F-2 male mice from birth to 8 weeks of age[a].

Age	Body weight (Grams)				
	Group C	Group A	Group A'	Group G	Group G'
Day 1	1.44 ± 0.10	1.37 ± 0.16	1.25 ± 0.17	1.44 ± 0.19	1.35 ± 0.15
Day 4	2.66 ± 0.28	2.45 ± 0.36	2.06 ± 0.42	2.69 ± 0.45	2.48 ± 0.30
Week 1	4.37 ± 0.38	3.91 ± 0.41	3.38 ± 0.49	4.43 ± 0.60	4.08 ± 0.32
Week 2	7.82 ± 0.56	7.26 ± 0.50	6.32 ± 0.91	8.13 ± 1.32	7.64 ± 0.36
Week 3	10.52 ± 0.96	9.34 ± 1.53	8.44 ± 0.74	10.20 ± 1.30	9.75 ± 1.09
Week 4	15.30 ± 1.33	13.80 ± 2.18	12.09 ± 0.84	14.62 ± 1.91	14.73 ± 1.38
Week 5	20.80 ± 1.59	18.66 ± 1.93	17.78 ± 1.22	19.30 ± 1.86	19.64 ± 1.48
Week 6	23.02 ± 2.13	20.56 ± 1.61	19.61 ± 1.28	21.54 ± 2.15	21.26 ± 1.41
Week 7	24.54 ± 2.52	22.17 ± 1.68	21.32 ± 1.48	23.35 ± 2.49	22.78 ± 1.93
Week 8	25.70 ± 2.73	23.48 ± 1.87	21.91 ± 1.13	24.27 ± 2.21	23.68 ± 1.33

[a] Data presented as mean ± standard deviation.

APPENDIX 25.

APPENDIX 25.

Table 31. Liver, thymus and testis weights of F-2 males[a].

	Group C	Group A	Group A'	Group G	Group G'
5 weeks of age					
Liver weight (Grams)	1.07 ± 0.08	1.00 ± 0.08	1.15 ± 0.09	1.24[c] ± 0.17	1.27[c] ± 0.04
(%)[b]	(100.0)	(93.5)	(107.0)	(116.2)	(118.9)
Liver/body weight ratio	0.053	0.057	0.063	0.063	0.061
(%)	(100.0)	(108.7)	(119.2)	(120.3)	(116.0)
Thymus weight (Milligrams)	48.2 ± 13.4	45.8 ± 20.4	34.1 ± 15.1	38.7 ± 4.5	54.2 ± 8.3
(%)	(100.0)	(94.9)	(70.7)	(80.3)	(112.3)
Thymus/body weight ratio	2.38×10^{-3}	2.63×10^{-3}	1.87×10^{-3}	1.98×10^{-3}	2.60×10^{-3}
(%)	(100.0)	(110.9)	(78.8)	(83.2)	(109.5)
Testis weight (Grams)	0.12 ± 0.00	0.10 ± 0.03	0.12 ± 0.02	0.16[d] ± 0.04	0.16[d] ± 0.02
(%)	(100.0)	(79.6)	(95.9)	(127.9)	(126.5)
Testis/body weight ratio	0.0060	0.0056	0.0065	0.0080	0.0075
(%)	(100.0)	(93.0)	(106.9)	(132.4)	(123.4)
Sample size	3	3	3	3	3
6 weeks of age					
Liver weight (Grams)	1.17 ± 0.09	1.41 ± 0.22	1.37 ± 0.13	1.29 ± 0.16	1.44 ± 0.15
(%)[b]	(100.0)	(119.7)	(116.8)	(109.9)	(122.4)
Liver/body weight ratio	0.052	0.065	0.068	0.063	0.064
(%)	(100.0)	(125.0)	(131.1)	(121.0)	(123.2)
Thymus weight (Milligrams)	50.1 ± 12.4	65.4 ± 3.6	61.7 ± 4.7	61.4 ± 13.9	62.9 ± 13.0
	(100.0)	(130.5)	(123.2)	(122.6)	(125.6)
(%)	2.20×10^{-3}	3.00×10^{-3}	3.05×10^{-3}	2.97×10^{-3}	2.79×10^{-3}
Thymus/body weight ratio	(100.0)	(136.3)	(138.3)	(134.9)	(126.5)
(%)	0.16 ± 0.02	0.16 ± 0.03	0.14 ± 0.01	0.16 ± 0.03	0.17 ± 0.03
Testis weight (Grams)	(100.0)	(98.0)	(87.8)	(95.9)	(104.1)
(%)	0.0072	0.0073	0.0071	0.0076	0.0075
Testis/body weight ratio	(100.0)	(102.3)	(98.5)	(105.6)	(104.8)
(%)	3	3	3	3	3
Sample size					

Table 31 (cont'd)

7 weeks of age					
Liver weight (Grams)	1.29 ± 0.13	1.45 ± 0.28	1.36 ± 0.15	1.34 ± 0.12	1.42 ± 0.15
(%)[b]	(100.0)	(112.7)	(106.0)	(104.4)	(110.4)
Liver/body weight ratio	0.053	0.064	0.062	0.061	0.061
(%)	(100.0)	(121.5)	(117.0)	(115.3)	(114.8)
Thymus weight (Milligrams)	41.2 ± 4.2	50.7 ± 4.5	65.3[d] ± 2.4	42.5 ± 9.0	56.7[d] ± 9.2
(%)	(100.0)	(123.1)	(158.5)	(103.2)	(137.5)
Thymus/body weight ratio	1.70×10^{-3}	2.25×10^{-3}	2.97×10^{-3}	1.93×10^{-3}	2.43×10^{-3}
(%)	(100.0)	(132.7)	(175.0)	(113.9)	(143.1)
Testis weight (Grams)	0.19 ± 0.04	0.20 ± 0.02	0.16 ± 0.03	0.17 ± 0.02	0.19 ± 0.04
(%)	(100.0)	(105.4)	(87.5)	(89.3)	(100.0)
Testis/body weight ratio	0.0077	0.0087	0.0074	0.0076	0.0080
(%)	(100.0)	(113.6)	(96.6)	(98.6)	(104.0)
Sample size	3	3	3	3	3
8 weeks of age					
Liver weight (Grams)	1.42 ± 0.23	1.56 ± 0.18	1.51 ± 0.16	1.69[d] ± 0.24	1.72[d] ± 0.18
(%)[b]	(100.0)	(109.8)	(106.8)	(119.0)	(121.0)
Liver/body weight ratio	0.055	0.066	0.069	0.069	0.074
(%)	(100.0)	(120.3)	(125.6)	(126.8)	(136.2)
Thymus weight (Milligrams)	37.5 ± 8.0	41.8 ± 16.5	33.0 ± 10.1	42.7 ± 9.4	36.1 ± 11.3
(%)	(100.0)	(111.5)	(88.1)	(114.0)	(96.3)
Thymus/body weight ratio	1.45×10^{-3}	1.76×10^{-3}	1.49×10^{-3}	1.75×10^{-3}	1.56×10^{-3}
(%)	(100.0)	(121.8)	(103.3)	(121.1)	(108.1)
Testis weight (Grams)	0.18 ± 0.02	0.18 ± 0.02	0.17 ± 0.02	0.19[f] ± 0.02	0.17 ± 0.03
(%)	(100.0)	(96.9)	(94.3)	(105.6)	(92.5)
Testis/body weight ratio	0.0070	0.0074	0.0078	0.0079	0.0073
(%)	(100.0)	(105.9)	(110.7)	(112.2)	(103.9)
Sample size	3	3	3	3	3

[a] Data presented as mean ± standard deviation.
[b] % refers to percent of Group C.
[c] Significantly different from Group C, $p<0.05$.
[d] Significantly different from Groups C and A, $p<0.05$.
[e] Significantly different from Groups C and G, $p<0.05$.
[f] Significantly different from Groups A' and G', $p<0.05$.

APPENDIX 26.

Table 32. Liver vitamin A and E concentrations in F-2 males[a,b,c].

	Group C	Group A	Group A'	Group G	Group G'
Retinol (%)[d]	994.3 (100.0)	199.0 (20.0)	134.0 (13.5)	502.7 (50.6)	423.3 (42.6)
Retinyl Palmitate (%)	216.7 (100.0)	87.5 (40.4)	8.2 (3.8)	126.3 (58.3)	46.7 (21.6)
Total Vitamin A (%)	1211.0 (100.0)	286.5 (23.7)	163.7 (13.5)	629.0 (51.9)	470.0 (38.8)
Vitamin E (%)	13.02 (100.0)	20.33 (156.1)	7.52 (57.8)	5.27 (40.5)	2.79 (21.4)
Sample size	1	1	1	1	1

[a] Vitamin A and E analyses were determined by Dr. Stowe's lab., the Animal Health Diagnostic Lab., Nutrition Section, MSU.
[b] Data expressed as μg/g of dry liver weight.
[c] F-2 males were 8 weeks old.
[d] % refers to percent of Group C.

APPENDIX 27.

Table 33. Caudal epididymal sperm concentrations of F-2 males at 6, 7, and 8 weeks of age[a,b].

	Sperm concentration (million/ml)				
Age	Group C	Group A	Group A'	Group G	Group G'
6 weeks	2.35 ± 1.28	1.75 ± 0.48	4.01 ± 2.05	2.73 ± 0.97	4.34 ± 0.96
7 weeks	7.69 ± 4.79	6.74 ± 2.92	7.16 ± 3.65	9.54 ± 4.64	6.36 ± 1.70
8 weeks	9.60 ± 0.94	6.15 ± 1.36	6.03 ± 3.64	5.35 ± 1.37	6.76 ± 4.62
Sample size	3	3	3	3	3

[a] Data presented as mean ± standard deviation.

[b] Sperm suspension was collected as followings: both of the epididymides were placed in a Falcon Organ Tissue Culture Dish (60 x 15 mm, Becton Dickinson Labware) and poked with a 25 G needle to release the sperm into 1.0 ml BMOC-3 medium. After a 30-min incubation at 37°C, 5% CO_2 in air and 100% humidity, 20 μl of the sperm suspensions were placed on a CellSoft 20μ-chamber and examined under a CellSoft Video-micrographic Computer-Assisted Semen Analyzer (CRYO Resources Inc., New York, NY). A minimum of 100 sperm cells were analyzed for each sample.

APPENDIX 28.

Table 34. Epididymal sperm motion quality of F-2 males[a,b].

Parameters[c]	Group C	Group A	Group A'	Group G	Group G'
6 weeks of age					
% motile sperm	36.1 ± 11.3	46.0 ± 10.7	30.6 ± 9.7	57.3 ± 12.3	58.3 ± 12.5
Velocity	155.3 ± 33.9	193.8 ± 174.7	139.3 ± 19.0	136.6 ± 30.7	141.0 ± 37.0
Linearity	6.2 ± 0.4	6.1 ± 0.2	4.6 ± 1.7	5.9 ± 1.8	5.7 ± 1.0
A.L.H.[d]	1.9 ± 1.7	1.8 ± 0.8	3.0 ± 3.6	3.1 ± 2.2	3.1 ± 1.0
B./C. F.[e]	9.5 ± 8.6	13.1 ± 0.1	7.9 ± 7.0	14.4 ± 5.1	12.9 ± 1.2
Sample size	3	3	3	3	3
7 weeks of age					
% motile sperm	47.5 ± 25.3	63.1 ± 12.0	66.3 ± 5.9	69.3 ± 16.7	68.1 ± 11.6
Velocity	170.9 ± 17.5	251.3 ± 14.9	222.0 ± 50.2	230.0 ± 58.6	208.4 ± 42.1
Linearity	4.3 ± 0.3	5.3 ± 0.7	5.2 ± 0.3	5.1 ± 0.6	5.4 ± 0.8
A.L.H.	4.6 ± 1.2	9.8 ± 0.8	6.9 ± 1.4	8.6 ± 4.0	2.3 ± 3.1
B./C. F.	13.4 ± 0.2	11.2 ± 1.4	10.0 ± 1.4	12.6 ± 0.2	13.0 ± 2.1
Sample size	3	3	3	3	3
8 weeks of age					
% motile sperm	65.0 ± 10.3	49.3 ± 17.7	55.3 ± 1.9	62.0 ± 8.1	49.4 ± 11.2
Velocity	170.6 ± 13.2	143.7 ± 23.7	151.3 ± 12.7	159.8 ± 47.5	166.4 ± 19.6
Linearity	3.9 ± 0.9	4.4 ± 0.8	4.6 ± 0.8	5.5 ± 0.9	4.9 ± 0.2
A.L.H.	5.1 ± 1.0	4.1 ± 1.0	4.6 ± 1.1	6.6 ± 1.2	4.8 ± 3.4
B./C. F.	11.5 ± 1.2	10.4 ± 0.8	12.4 ± 1.0	12.3 ± 2.1	11.4 ± 1.1
Sample size	3	3	3	3	3

[a] For sperm motion quality analysis, 20 μl of the sperm suspension were placed on a CellSoft 20 μ chamber and examined under a CellSoft Video-micrographic Computer-Assisted Semen Analyzer (CRYO Resources Inc., New York, NY). A minimum of 100 sperm cells were analyzed for each sample. Sperm suspension was collected from treated male epididymides and had been incubated in BMOC-3 medium for one half-hour at 37°C, 5% CO_2 in air, 100% humidity.

[b] Data presented as mean ± standard deviation.
No statistical significance was observed among the groups, $p > 0.05$.

[c] See Appendix 3 for definition of parameters.

[d] A.L.H. represents amplitude of lateral head displacement.

[e] B./C. F. represents beat/cross frequency.

APPENDIX 29.

Table 35. Reproductive performance of F-2 males[a,b].

Parameters	Group C	Group A	Group A'	Group G	Group G'
In vitro fertilizing ability of epididymal sperm[c]					
No. of oocyte tested	41.3 ± 4.9	24.7 ± 6.0	33.7 ± 8.0	25.0 ± 4.4	25.7 ± 5.7
IVF[d] rate (%)	91.4 ± 9.0	82.3 ± 18.5	65.2 ± 19.8	83.8 ± 4.8	81.6 ± 24.2
Sample size	3	3	3	3	3
Breeding ability[e]					
Fecundity[f]	4/6	6/6	6/6	6/6	6/6
Litter size	11.5 ± 1.2	9.5 ± 2.0	8.6 ± 0.5	8.8 ± 1.9	9.2 ± 2.5
Viability[g]	89.5 ± 7.7	92.4 ± 8.9	68.1 ± 80.1	91.6 ± 6.8	74.2 ± 16.5
Body weight at 7 days of age (gm)	4.31 ± 0.34	4.11 ± 0.44	3.56[h] ± 0.37	4.70 ± 0.49	3.69[h] ± 0.56

[a] Data presented as mean ± standard deviation.
[b] For *in vitro* fertilization, F-2 males were 8 weeks old, whereas, for breeding, the mice were 7 weeks old.
[c] To determine the sperm *in vitro* fertilizing ability, sperm was used to inseminate oocytes which were collected from PMSG/hCG superovulated previously non-treated females. The sperm suspension was collected from treated male epididymides and had been incubated in BMOC-3 medium for one half-hour at 37°C, 5% CO_2 in air, 100% humidity. Oocytes were inseminated with 50 μl of sperm suspension (1-3 x 10^7) and were co-incubated at 37°C, 5% CO_2 in air, 100% humidity for 24 hours before examination of fertilization.
[d] IVF refers to *in vitro* fertilization.
[e] For breeding, 7 F-1 males were paired each with a previously non-treated mature female. The females began to receive the same treatment diets as the males when they were housed together for 5 days. These females were then housed individually and continuously provided with treatment diets through gestation and lactation.
[f] Fecundity refers to number of females giving birth/number of females bred.
[g] Viability refers to percent of F-2 mice that survived to the age of 7 days.
[h] Significantly different from Groups C, A and G, $p<0.05$.

APPENDIX 30.

Table 36. Body, liver and thymus weights of F-2 females[a,b].

Parameters	Group C	Group A	Group A'	Group G	Group G'
Body weight					
Grams	17.8 ± 2.1	17.0 ± 2.7	16.0[d] ± 2.0	18.6 ± 2.2	17.2 ± 1.6
(%)[c]	(100.0)	(95.5)	(89.9)	(104.5)	(96.6)
Liver weight					
Grams	1.09 ± 0.13	1.22 ± 0.22	1.10 ± 0.17	1.31[e] ± 0.23	1.24 ± 0.14
(%)	(100.0)	(111.9)	(100.9)	(120.2)	(113.8)
Liver/body weight ratio	0.061	0.072[f]	0.069[f]	0.070[f]	0.072[f]
(%)	(100.0)	(118.0)	(113.1)	(114.8)	(118.0)
Thymus weight					
Milligrams	56.5 ± 17.4	57.4 ± 16.4	63.7 ± 18.0	65.0 ± 14.4	57.0 ± 13.6
(%)	(100.0)	(101.6)	(112.7)	(115.1)	(100.9)
Sample size	15	15	15	15	15

[a] Data presented as mean ± standard deviation.
[b] F-2 females were between 31 to 44 days old.
[c] % refers to percent of Group C.
[d] Significantly different from Group G, $p<0.05$.
[e] Significantly different from Groups C and A, $p<0.05$.
[f] Significantly different from Group C, $p<0.05$.

APPENDIX 31.

Table 37. Liver vitamin A and E concentrations in F-2 females[a,b,c].

	Group C	Group A	Group A'	Group G	Group G'
Retinol	784.0	328.3	155.0	540.0	300.3
(%)[d]	(100.0)	(41.9)	(19.8)	(68.9)	(38.3)
Retinyl Palmitate	99.7	18.0	9.3	36.3	29.0
(%)	(100.0)	(18.1)	(9.3)	(36.4)	(29.1)
Total Vitamin A	883.7	346.3	164.3	576.3	329.3
(%)	(100.0)	(39.2)	(18.6)	(65.2)	(37.3)
Vitamin E	22.56	3.43	3.99	8.49	5.98
(%)	(100.0)	(15.2)	(17.7)	(37.6)	(26.5)
Sample size	3	3	3	3	3

[a] Vitamin A and E analyses were determined by Dr. Stowe's lab., the Animal Health Diagnostic Lab., Nutrition Section, MSU.
[b] Data expressed as μg/g of dry liver weight.
[c] F-2 males were 8 weeks old.
[d] % refers to percent of Group C.

APPENDIX 32.

Table 38. *In vitro* fertilizing ability of F-2 females[a].

Parameters	Group C	Group A	Group A'	Group G	Group G'
Superovulation rate[b]	11/11	9/10	10/12	10/11	11/12
No. of oocyte ovulated[c]	29.5 ± 18.5	16.7 ± 9.9[d]	17.2 ± 6.8[d]	20.1 ± 17.8	17.8 ± 9.1[d]
% oocytes fertilized[c]	81.2 ± 26.0	77.8 ± 21.7	84.1 ± 15.7	75.1 ± 15.8	87.7 ± 9.3
Sample size	11	10	12	11	12

[a] To determine the oocyte *in vitro* fertilizing ability, oocytes were inseminated with sperm from previously non-treated males. Oocytes were collected from PMSG/hCG superovulated treated females. The ages were between 31 to 44 days old. The sperm suspension was collected from mature male epididymides and had been incubated in BMOC-3 medium for one half-hour at 37°C, 5% CO_2 in air, 100% humidity.
Oocytes were inseminated with 50 μl of sperm suspension (1-3 x 10^7) and were co-incubated at 37°C, 5% CO_2 in air, 100% humidity for 24 hours before examination for fertilization.

[b] Superovulation rate refers to number of females ovulated/number of females superovulated with PMSG/hCG.

[c] Data presented as mean ± standard deviation.

[d] significantly different from Group C, $p<0.05$.

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