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INDUCTION OF SQUAMOUS CELL CARCINOMA IN THE MANDIBLE AND MAXILLA, AND REPRODUCTIVE DYSFUNCTION IN MINK (MUSTELA VISON) CAUSED BY 3,3',4,4',5-PENTACHLOROBIPHENYL (PCB 126)

presented by

Kerrie J. Beckett

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Major Professor's Signature

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INDUCTION OF SQUAMOUS CELL CARCINOMA IN THE MANDIBLE AND MAXILLA, AND REPRODUCTIVE DYSFUNCTION IN MINK (*MUSTELA VISON*) CAUSED BY 3,3',4,4',5–PENTACHLOROBIPHENYL (PCB 126)

By

Kerrie J. Beckett

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ABSTRACT

INDUCTION OF SQUAMOUS CELL CARCINOMA IN THE MANDIBLE AND MAXILLA, AND REPRODUCTIVE DYSFUNCTION IN MINK (*MUSTELA VISON*) CAUSED BY 3,3',4,4',5–PENTACHLOROBIPHENYL (PCB 126)

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The hypothesis of this dissertation is that the lesion induced by 3,3',4,4',5-pentachlorobiphenyl (PCB 126), identified as squamous epithelial proliferation in the maxilla and mandible of mink (*Mustela vison*), is oral squamous cell carcinoma. The objectives of this study were to determine the progression of the lesion after removal of the stimulus (PCB 126), and the ability of the cells comprising the lesion to form a tumor after being transplanted or injected into nude, athymic mice. The lesion was histologically detectable in mink exposed to 24 μg PCB 126/kg feed for one to six weeks, and was clinically detectable in the PCB 126-exposed mink of the three-, four-, and five-week groups, by 12-weeks post-treatment. The lesion worsened during the six- or eight-month post-exposure period in 100% of the mink in all exposure groups, thus meeting the first criteria of lesion progression after withdrawal of the PCB 126 stimulus. In the athymic mouse trial, nodular growths were observed in two of the 18 mice that were implanted with gingiva from the oral lesion of PCB 126-exposed mink. Although the tumors in the mice were classified as non-malignant, the origin was non-host (mink) stratified squamous epithelium, demonstrating consistency with neoplastic criteria. Another objective of the study was to determine if the oral neoplasia caused

systemic osteolysis in addition to local osteolysis of the jawbones in PCB 126-exposed mink. A series of radiographs evaluated dental alignment, bone lysis, nail growth, organ integrity and seven skull and subcutaneous fat measurements of PCB 126-exposed and control mink. In addition, calcium and phosphorus content of femurs were assessed. Radiographs revealed that PCB 126 caused severe localized mandibular and maxillary osteolysis that aggressively progressed into the zygoma and nasal turbinates. However, systemic effects of PCB 126 on bone were not observed in this study, and there were no significant changes in femur calcium or phosphorus content. In addition to the oral lesion assessments, a study was conducted to determine the reproductive effects of PCB 126 in female mink. No reproductive effects were observed at 0.24 μg PCB 126/kg feed. However, total reproductive failure occurred at dietary concentrations as low as 2.4 μg PCB 126/kg feed. A number of changes in clinical, hematological and serum chemistry parameters were observed in both the adult female and juvenile mink exposed to PCB 126.

Wild mink naturally exposed to environmental contaminants were also assessed for the prevalence of the oral neoplasia. Four of nine mink trapped in a PCB-contaminated, Superfund site exhibited histological evidence of the oral lesion. There was a significant correlation between the severity of the lesion and hepatic concentrations of total PCBs and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin toxic equivalents in these mink. Results from these experiments indicate that PCB 126 induces an extremely invasive and destructive form of oral squamous cell carcinoma, as well as other adverse effects in mink.

DEDICATION

~ To Mom ~

&

~ My Co-Pilots ~

~

"If I could wish for anything,
I should not wish for wealth and power,
but for the passionate sense of what can be,
for the eye, which, ever young and ardent sees the possible."

- Soren Kierkegaard

~ Thank You ~

For seeing in me what others never did, and
Instilling in me the Passion for Life,
and
Following my Dreams and the Possibilities.

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I would like to take this opportunity to acknowledge those that have been with me and supported me in so many different facets on this journey of graduate school. So to my committee: to my advisor, Dr. Steve Bursian, I express my appreciation for your guidance and friendship. Drs. Ben Yamini, Mike Orth, Diana Rosenstein and Jim Sikarski, the team that has offered their support and expertise during my program at MSU. Dr. Richard Aulerich deserves special thanks as an honorary committee member and mentor. It goes without saying that he has been a rock of support and knowledge for me and the kindest of friends, and also the man that introduced me to mink.

Before I go any further, this research would not have been possible without the mink over the years (most of whom were named, and listing them would take up several pages in this acknowledgment). And to those of you who know me best, it would be most appropriate for the animals to come first.

The Michigan State University (MSU) Fur Farm has been a fabulous opportunity for toxicology research, and a special thanks goes out to the crew at the farm for their help, even when we got sprayed by the mink: Angelo Napolitano, Jeff Greenlee, and especially Dianne Karsten. In addition, thanks to Bob Kriegel, Jackie Christie, and Betty Walther, your help and friendship have been immeasurable.

With that said, my pillar of support: my mom, you're the best. Thank you for always being there for me, and giving me the strength and courage to do whatever it was that I set out to do. I would also like to thank my sister Kris, (and not necessarily for those annoying little lectures along the way). My beloved pets: Harley, my co-pilot -

always my calm in the storm. The dogs, cats, and Petie are always the ones who understand without expectation or judgment, and who have always greeted me warmly after an unspeakably stressful day of graduate school (to say the least).

Learning as a graduate student only begins at the book level. Learning becomes significant when we are excited by what we learn, and we become creative in our learning. Struggling, coping, stressing-out, and remembering how to laugh are lessons that are vital to learning in graduate school. It is how you put them together in a package that is important. Thanks to ALL of you who have been there for me, as I have also learned a part of my education from you.

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LIST OF ABBREVIATIONS

AB	alveolar bone
AG	anion gap
AHH	aryl hydrocarbon hydrolase
AhR	aryl hydrocarbon receptor
ALB	albumin concentration
ALK PHOS	alkaline phosphatase activity
ALT	alanine aminotransferase activity
AMYL	amylase activity
ARNT	aryl hydrocarbon receptor nuclear translocator
AST	aspartate aminotransferase activity
bHLH	basic helix-loop-helix
BUN	blood urea nitrogen concentration
BW	body weight
C	canine teeth
Ca	calcium concentration
CHOL	cholesterol concentration
CK	creatinine kinase activity
Cl	chloride concentration
CO_2	carbon dioxide
CREAT	creatinine concentration
CYP1A1	cytochrome P-450 1A protein/enzyme; the major hydrocarbon inducible form of the P-450 biotransformation enzyme

DDT	1,1,1-trichloro-2,2-*bis*(p-chlorophenyl)ethane
DNA	deoxyribonucleic acid
DRE	dioxin-response-element
DV	dorso-ventral view
Eagle's ASP	Eagle's minimum essential solution
EDTA	ethylenediaminetetraacidic acid
EGF	epidermal growth factor
ELISA	enzyme-linked-immunosorbent-assay
FA	fluctuating asymmetry
FCRA	Fort Custer Recreation Area
Fe	iron concentration, serum
gamma-GTP	gamma-glutamyl transpeptidase activity
Gin; GIN	gingiva
GJIC	gap junctional intercellular communication
GLOB	globulin concentration
GLUC	glucose concentration
GPx	glutathione peroxidase activity
GR	GSH reductase activity
GSH	glutathione concentration
H	height
H & E	hematoxylin and eosin
HCT	hematocrit
HGB	hemoglobin concentration,

hsp90	heatshock proteins
I	incisor teeth
i.d.	internal diameter
IARC	International Agency for Research on Cancer
K	potassium concentration
KRAOC	Kalamazoo River area of concern
kVp	kilivolt peak
L	length
LD_{50}	median lethal dose
LOAEL	lowest observed adverse effect level
M	molar teeth
mAs	milliamps per second
MCHC	mean corpuscular hemoglobin concentration
MCV	mean corpuscular volume
MEM	Modified Eagle's Medium solution
Mg	magnesium concentration
mRNA	messenger ribonucleic acid
MSU	Michigan State University
n	number
NA	not available
Na	sodium concentration
NOAEL	no observed adverse effect level
NTP	National Toxicology Program

OC	osteocalcin
p	level of significance, based on Type I error rate of $\alpha = 0.05$ ($p \leq 0.05$)
PAHs	polyhalogenated aromatic hydrocarbons
PBS	buffering solution
PCB 30	2,4,6-triphenyl
PCB 77	3,3',4,4'-tetrachlorobiphenyl
PCB 81	3,4,4',5-tetrachlorobiphenyl
PCB 126	3,3',4,4',5-pentachlorobiphenyl
PCB 136	2,2',3,3',6,6'-hexachlorobiphenyl
PCB 153	2,2',4,4',5,5'-hexachlorobiphenyl
PCB 169	3,3',4,4',5,5'-hexachlorobiphenyl
PCB 204	2,2',3,4,4',5,6,6'-octaphenyl
PCDDs	polychlorinated dibenzodioxins
PCDFs	polychlorinated dibenzofurans
PDL	periodontal ligament
PLT	platelet volume
PM	premolar teeth
r^2	coefficient of determination
RBC	red blood cell count
RDW	red cell distribution width
rpm	rotations per minute (centrifuge)
SAS	Statistical Analysis Systems software, version 8.0
SCC	squamous cell carcinoma

SDH	sorbitol dehydrogenase activity
SE	standard error of the mean
Se	Selenium concentration
SOT	squamous odontogenic tumor
T	tooth
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TCO_2	total carbon dioxide concentration, serum
TEF	toxic equivalency factor, or toxicity equivalency factor
TNF-α	tumor necrosis factor
TRVs	toxicity reference values
U.S.EPA	U.S. Environmental Protection Agency
W	width
WBC	white blood cell count
ZB-5	5% phenyl polysiloxane

Abbreviations for Units of Measure:

µg	microgram
µL	microliter
cm	centimeter
dL	deciliter
fL	femtoliter
g	gram
hr	hour

IU	International Units
kg	kilogram
km	kilometer
mg	milligram
millisec	milliseconds
min	minute
mL	milliliter
mm	millimeter
mM	millimolar
mos	milliosmole; unit used for osmolality, also written mOsm
ng	nanogram
pg	picogram

INTRODUCTION

The mink (*Mustela vison*) is a relevant wildlife species, and an important species in toxicology research. The mink is an indicator species of environmental contaminant exposure and ecosystem health, and has been approved by the United States Environmental Protection Agency (U.S. EPA) and recognized by the National Academy of Science as a preferred toxicological model (Calabrese et al. 1992). The mink is a highly sensitive species to environmental contaminants, especially polychlorinated biphenyls (PCBs). Mink are found throughout most of the northern U.S. and Canada and associated with semi-aquatic environments, as well as being apex predators. Being at the top of the food web, the mink has the potential to bioaccumulate environmental contaminants through the prey items it consumes. Polychlorinated biphenyls are ubiquitous environmental contaminants that are resistant to biological and chemical degradation, and thus can be encountered via prey items. The toxicity of these contaminants can give rise to serious and significant effects on animal and human health. The most toxic PCB congener is considered to be 3,3',4,4',5-pentachlorobiphenyl (PCB 126) and is known to be prevalent in the environment.

Our laboratory originally identified an oral lesion in mink kits and juvenile mink induced by PCB 126 and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). The lesion was initially identified as squamous epithelial proliferation of the mandible and maxilla, and was described as clumping of the incisors and thickening and nodular growths of the gingiva. Further studies indicated that kits and juvenile mink were not uniquely sensitive to the toxicity of PCB 126 in terms of the development of maxillary and

mandibular squamous proliferation as the endpoint, as the lesion was induced in adult female mink fed a diet containing PCB 126.

The oral lesion was the focus of this research. Therefore, a complete description of the lesion is important to understanding its progression. Initially, small islands of atypical squamous epithelial cells form within the gingiva in no particular location. Subsequently, these stratified squamous epithelial cells form large islands and develop into cysts. Some of these cysts may contain centers of exfoliated epithelia and possibly keratin. Nests and/or cords of cells infiltrate into the periodontal ligament and/or alveolar bone destroying the underlying tissue. This leads to periodontoclasia (loosening and displacement of teeth), and in severe cases, tooth loss. In addition, pathologic changes were observed as swollen, inflamed, and bleeding gingiva, including recession of the gums. Aphagia, defined as the inability to eat, often ensued at this stage, leading to a subsequent rapid weight loss.

Commonly accepted criteria for malignancy are: 1) tissue invasion (referred to as a hallmark of malignant neoplasia); 2) the lack of dependence on the continued presence of the stimulus (irreversible); 3) increased rate of tissue growth; 4) differentiation and anaplasia (with higher proliferative activity); and 5) metastasis (also considered a hallmark of malignancy). Metastases are not observed as frequently in animals as they are in humans, either due to the shorter life span of animals, biological reasons, or the fact that they are not screened for and thus, detected as a practice.

The mink is an important animal model for studying PCB 126-induced oral squamous cell carcinoma (SCC), and the pathological effects this PCB congener has on bone and possibly other biological systems. The overall hypothesis of the research

described in this dissertation is that the oral lesion that is induced by PCB 126 in the maxilla and mandible of mink is aggressive oral squamous cell carcinoma. The objectives of this research were to:

1) determine the reproductive effects of PCB 126 in female mink and its effects on kit survival;
2) determine the biological effects, as assessed by clinical, hematological, and serum chemistry parameters, in adult female and seven-month-old mink caused by consumption of PCB 126;
3) determine if the oral lesion in mink was malignant, by meeting two additional criteria of carcinogenicity, (a) the progression of the lesion after removal of the stimulus (PCB 126); and (b) the ability of the cells comprising the lesion to grow and form a tumor after being transplanted and/or injected into athymic mice;
4) determine if the oral neoplasia caused systemic osteolysis in addition to the observed local (maxillary and mandibular) osteolysis; and
5) determine the prevalence of the oral neoplasia in wild mink that were naturally exposed to environmental contaminants, and were trapped in the Kalamazoo River Basin, a designated Superfund site.

CHAPTER I

Literature Review

A. Environmental Contaminants

History of PCBs:

Polychlorinated biphenyls (PCBs) are recognized as environmental contaminants that are of concern. The history of PCBs started in 1929 when they were commercially produced because of their heat-insulating and physical stabilizing properties. Production of PCB industrial mixtures continued under the trade name Aroclor® by the major U.S. producer, Monsanto Corporation (St. Louis, MO). A variety of uses for PCBs included dielectric fluids in capacitors and transformers, hydraulic lubricants, organic diluents, plasticizers, pesticide extenders, adhesives, dust-reducing agents, cutting oils, flame retardants, sealants, printing inks, paints, and pigment in carbonless copy paper (Hutzinger et al. 1974; Safe 1990; Erickson 1997). Polychlorinated biphenyls also occurred as unintentional byproducts through synthesis of compounds, such as vinyl chloride, chlorinated benzenes, chloroform, and adhesives (Safe 1990; Erickson 1997). Production of PCBs was banned in North America and Western Europe in 1977, and Eastern Europe and Russia in the 1990s (Giesy and Kannan, 1998).

Disposal regulations were not implemented until after the impacts of PCBs had been recognized. Legal and illegal uses, as well as disposal, spills, and leaking of PCBs have contributed to their entry into the environment. Gradual advection of PCBs near

the source has contributed to low background level contamination in the environment (Safe 1990; Erickson 1997). The transport of PCBs in the environment is complex and global (Erickson 1997). The properties that made PCBs so appealing for industry are also the characteristics that are responsible for PCBs being a ubiquitous environmental contaminant.

Polychlorinated biphenyls are considered to be one of the most widespread and persistent anthropogenic contaminants in the global ecosystem giving rise to serious environmental contamination and creating a potential health hazard (Safe 1990; Nesaretnam et al. 1996; Wilson and Safe, 1998). The stability of PCBs has made them highly persistent in the environment, where degradation depends on the structural arrangement of the chlorines. Polychlorinated biphenyls have a tendency to bioaccumulate and biomagnify in the food chain, due to the lipophilicity of the chemicals (Safe 1990; Giesy and Kannan, 1998). Thus, PCBs are detected in every environmental compartment of the global ecosystem, including the air, water, soil/sediment, and biota such as fish, wildlife, and human adipose tissue, milk, and serum (Safe 1990; Safe 1993; Wilson and Safe, 1998).

PCB Classification:

Polychlorinated biphenyls belong to a larger class of chemicals known as polyhalogenated aromatic hydrocarbons (PAHs), which include polychlorinated dibenzo-*p*-dioxins, polychlorinated dibenzofurans, polychlorinated biphenyls, polychlorinated diphenyl ethers, and polychlorinated naphthalenes. Polychlorinated

biphenyls are formed through the process of chlorination, in which substitution reactions attach chlorines to the biphenyl rings.

The group of PCBs consisting of 209 discrete chemical compounds can exhibit different physiochemical and biochemical (e.g. metabolism and biodegradation) properties that result in environmental and biological breakdown. These molecular qualities of PCBs contribute to the analytical properties that can affect environmental transport and fate, bioconcentration factors, persistence and bioaccumulation. These physical properties of PCBs include molecular weight, vapor pressure, melting point, partition coefficient, aqueous solubility, sediment sorption properties, partitioning among gases (fugacity), and Henry's Law constants. Chemical properties can also include such parameters as density, viscosity, flash point, specific gravity, vaporization rate, dielectric constant, and solubility (in 25°C water) (Safe 1990; Erickson 1997).

These 209 individual PCB chemical compounds are called *congeners*. The term *homolog* is used when PCBs are subdivided by their degree of chlorination (the number of chlorines, which can range from one to ten, are distributed around the biphenyl). The chlorines are attached at one of the ten positions of the phenyl ring, identified as *ortho-*, *meta-*, and *para-* positions (Figures 1a and 1b). Polychlorinated biphenyls within a given homolog that present different positions of chlorine substitution are called *isomers* (Erickson 1997).

PCB Configuration:

Within the PCB structure, the two-phenyl moieties can rotate about the single 'bridge-bond'. Chlorine atoms in the *ortho* positions create more steric hindrance to the

rotation. PCB congeners with zero (or one) chlorine substitution in the *ortho* position lack steric hindrance, and therefore can assume a coplanar configuration (phenyl rings lying in the same plane). A few PCB congeners are capable of this coplanar configuration, but only four PCB congeners are non-*ortho* and are considered to be most like TCDD. These include 3,3',4,4',5-pentachlorobiphenyl (PCB 126), 3,3',4,4',5,5'-hexachlorobiphenyl (PCB 169), 3,3',4,4'-tetrachlorobiphenyl (PCB 77), and 3,4,4',5-tetrachlorobiphenyl (PCB 81). These congeners induce toxic responses similar to those reported for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Studies on the structure-function relationships have confirmed the similarities between TCDD toxicity and coplanar, non-*ortho* substituted PCB congener toxicity (Safe 1993; Erickson 1997).

The PCB congener PCB 126 is non-*ortho* substituted and is considered to be the most toxic PCB congener (Figure 2) (Van den Berg et al. 1998). PCB 126 is structurally similar to TCDD, and is one-tenth as toxic as TCDD (Van den Berg et al. 1998). PCB 126 assumes a coplanar configuration and induces toxic responses similar to those reported for TCDD, and PCB 126 is known to be prevalent in the environment (Safe et al. 1985; Safe 1990; Peterson et al. 1993). This coplanar configuration may be necessary for these PCB congeners and TCDD to competitively bind to a common receptor known as the aryl hydrocarbon receptor (AhR) that has been suggested as the mechanism of toxic action for these chemicals.

Figure 1-1a. The basic PCB structure: Two phenyl rings are connected, with chlorine atoms distributed around the biphenyl at the *ortho*, *meta*, and/or *meta* positions.

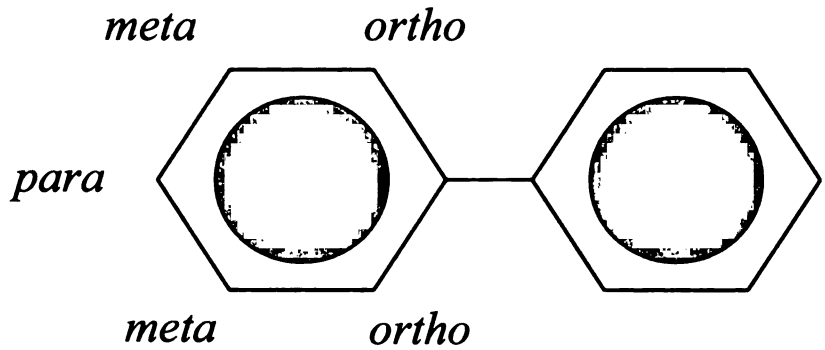


Figure 1-1b. A PCB has the basic formula of $C_{12} H_{10-n} Cl_n$, where n = 1 – 10.

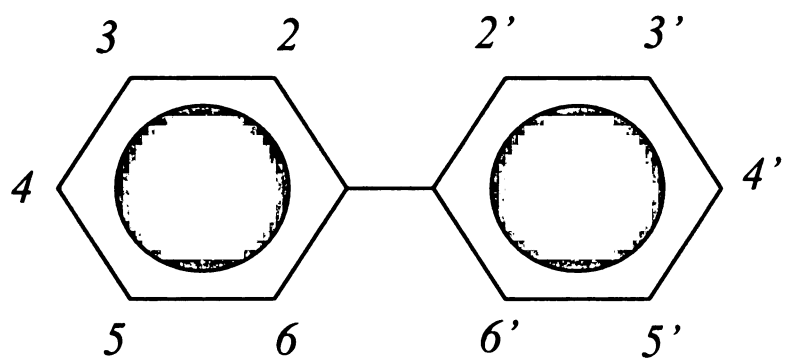
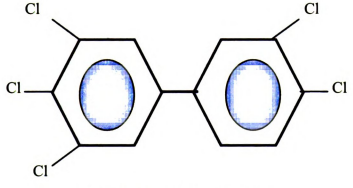


Figure 1-2. The chemical structure of 3,3',4,4',5-pentachlorobiphenyl (PCB 126).



3,3',4,4',5-Pentachlorobiphenyl
(PCB 126)

PCB Mechanism of Toxic Action:

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin and TCDD-like PCB congeners may act via a common mechanism of action, the Ah receptor. Initial studies by Poland et al. (1979), and a variety of studies that followed, including genetic, biochemical, and toxicological studies, lend support to the role that the AhR protein may play in mediating the toxic effects of TCDD and TCDD-like PCB congeners (Safe 1985; Safe 1988; Safe 1990; Wilson and Safe, 1998).

The AhR belongs to a group of proteins that contains a basic helix-loop-helix (bHLH) domain near the N-terminus, and thus can undergo dimerization and DNA-binding (Wilson and Safe, 1998). The AhR is a ligand-activated transcription factor that has no known endogenous, or naturally occurring ligand (Safe 1990; Josephy 1997; Giesy and Kannan, 1998). The AhR is the only known protein in its superfamily that requires an exogenous ligand for activation (Safe 1990; Wilson Safe, 1998). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin binds to the AhR with high affinity, and has been identified as the "prototype ligand" of the Ah receptor. In addition, the AhR appears to bind several different structural classes of chemicals that are considered to be AhR agonists, including halogenated aromatics, in addition to TCDD (Safe 1990; Yamaguchi et al. 1997).

Prior to binding of the ligand (e.g. TCDD), the unoccupied cytosolic AhR is normally complexed in a 1:2 ratio with heatshock proteins (hsp90). The hsp90 dissociate when the ligand binds to the AhR, enabling the receptor to be phosphorylated by tyrosine kinase. The activated AhR then enters the nucleus and forms a heterodimer complex with the Ah-receptor-nuclear translocator (ARNT). Inside the nucleus, the

AhR-ARNT complex binds to a regulatory sequence, known as the dioxin-response-element (DRE), and enhances transcription of the CYP1A1 gene and other genes with a DRE or DRE-like sequence in the upstream enhancer region (i.e. glutathione-S-transferase). The DRE is only a small segment of DNA (the consensus sequence is 5'-TXGCGTG-3' [X=T or A], in the 5'-flanking regions of the responsive genes), which can be located more than 1000 bases from the initiation site for transcription. The enhancer region of the CYP1A1 gene contains multiple DREs, which account for the marked increase (<100 fold) in CYP1A1 mRNA and protein levels following exposure to ligands for AhR (Safe 1988; Safe 1990; Safe 1993; Josephy 1997; Wilson and Safe, 1998).

The ARNT was initially thought to be a cytosolic protein that simply facilitated the translocation of the ligand-bound AhR into the nucleus. It is now recognized as an important component of the receptor complex that binds to DNA and activates transcription of genes under the control of AhR. The AhR complex has the ability to act as a ligand-dependent DNA-binding transactivator of gene expression (Schmidt and Bradfield, 1996; Rowlands and Gustafsson, 1997; Wilson and Safe, 1998).

Toxic Responses of PCBs:

A plethora of adverse effects have been attributed to PCBs. Studies have reported enzyme induction, thymic involution and atrophy, immunotoxicity, hyperkeratosis, dermal toxicity, cleft palate, hydronephrosis, hepatotoxicity, teratogenesis, developmental and reproductive toxicity, endocrine effects, neurotoxicity,

carcinogenicity, and lethality (Kociba 1978; Safe 1993; Abbott et al. 1994; Safe 1994; Erickson 1997; Giesy and Kannan, 1998).

As stated above, the toxic effects of a PCB congener are directly related to its structure. Therefore, the substitution and arrangement of chlorines around the biphenyl dictate the toxicity of the chemical. The toxic effects of a chemical are also mediated through a mechanism of action, which may or may not be common to sub-classes of congeners. The structural class of PCB congeners that are not TCDD-like based on their chlorine substitution, appear to exhibit adverse effects including neurotoxicity, estrogenicity, and protein-binding activities (Erickson 1997; Giesy and Kannan, 1998). PCBs have been extensively studied in both *in vivo* animal studies and *in vitro* assays (Erickson 1997). The majority of the studies have used commercial mixtures of PCBs, and only a minimal number have been conducted using individual PCB congeners.

Exposure to PCBs:

As a result of toxicity potential, widespread distribution, persistency, and potential for bioaccumulation, PCBs are considered a health concern. Polychlorinated biphenyls are lipophilic and resistant to biochemical degradation, therefore they have the ability to biomagnify in the food chain. This can result in an increase in the exposure potential for apex predators including humans. Polychlorinated biphenyl concentrations in food (prey), and therefore, tend to correlate with the fat content of that food source (Erickson 1997).

Toxic Differences Between Species:

Species, strain, as well as age, sex, reproductive status, and conditions of exposure (concentration, duration, and frequency) can influence the toxic effects of PCBs. When comparing species typically used in PCB toxicology studies, i.e. rats, guinea pigs, hamsters, and mink, a pronounced sensitivity to PAHs varies over a 5000-fold range between these species and strain, with guinea pigs being the most sensitive and the hamster being the least sensitive (Poland and Knutson 1982; Safe 1990; Josephy 1997). For example, the reported oral LD_{50} for TCDD in the guinea pig is approximately 1.0 μg/kg body weight (BW). The oral LD_{50} for the rat is reported at 10 to several 1000-fold higher than the guinea pig, depending on sex and strain (Josephy 1997).

The biological response an animal will have following exposure to PCBs that is measured as an endpoint is influenced by these characteristics, i.e., sex, age, reproductive status, strain, and species (Josephy 1997). A specific endpoint in one species may be a good indicator of a toxic response, but not a good indicator in another species. Thus, based on species specificity and species differences, not all species respond the same. For example, Hori et al. (1997) reported on the different species mechanisms by which PCB 126 induced toxicity in rats. Selenium (Se) and glutathione (GSH) concentrations, and glutathione peroxidase (GPx), GSH reductase (GR) and gamma-glutamyl transpeptidase (gamma-GTP) activities were significantly decreased by PCB 126 treatment in rat liver (Hori et al. 1997). In hepatic tissue of the guinea pig, GPx activity was significantly increased by PCB 126 exposure. No effect on Se, GSH or the enzyme activities was observed in guinea pigs (Hori et al. 1997).

B. Mink (*Mustela vison*):

Description:

Mink (*Mustela vison*) belong to the order Carnivora and the family Mustelidae, which also includes four subfamilies: Mephitinae (skunks), Taxidinae (badgers), Lutrinae (otters), and Mustelinae (martens, polecats, ferrets, weasels, and mink), comprised of 25 genera (Calabrese et al. 1992; Aulerich et al. 1999).

Wild mink are dark brown in color, sometimes with a white throat patch. Their fur is extremely dense and silky-soft. They have elongated bodies that are "weasel-like", with relatively short legs. Their tail measures about one-third of the body length and is somewhat bushy. Females are approximately one-half to two-thirds the size of the males. Females may weigh 600 g and males weigh about 1000 g (wild mink). A feature common to the Mustelidae is the presence of anal glands, which produce a distinct odor. These glands are derived from apocrine glands, and are important in defense, recognition, territorial marking, and courtship behavior (Smith and Schenk, 2000). The mink is very agile and quick, an excellent swimmer, and has powerful jaws. These are characteristics that enable the mink to be an efficient predator.

Mink are a semi-aquatic species, inhabiting the margins of aqueous habitats, i.e. rivers, ponds, marshes, lakes, and estuarines that extend throughout most of the northern parts of North America. Mink are considered solitary by nature and are generally nocturnal. Mink are piscivorous mammals and are considered to be true predators. Thus, mink are apex predators and placed at the peak of the food chain. Therefore, mink are potentially exposed to a wide variety of environmental contaminants through consumption of contaminated prey.

Mink were trapped for their fine quality of fur. Concern that the wild stock was being depleted prompted people to attempt raising mink by the end of the nineteenth century (Bowness 1996). However, successful mink ranching as a commercial practice is a relatively recent development and has occurred only within the last 80 years (Calabrese et al. 1992; Bowness 1996). The information that exists on the mink (biology, health, and husbandry) is more or less a direct result of the commercial ranching of mink. For example, bringing solitary animals into close contact with one another presented health risks, and as a result, vaccinations have been developed to reduce the incidence of diseases in mink (Freeman 1996). Because mink are one of the most recent species to be domesticated, their nutritional requirements needed to be identified and met in a commercial setting (NRC 1982; Atkinson 1996). Meeting nutritional requirements, especially during vulnerable periods of growth and development, and during high demand periods such as lactation, is essential for the health and well-being of the mink (Atkinson 1996; Aulerich et al. 1999). Without proper balancing of nutrition, deficiencies may lead to additional health problems such as wet belly, urolithiasis (with associated uroliths), steatitis (Vitamin E deficiency), cotton fur (iron deficiency), fur loss and depigmentation (biotin deficiency) and convulsions with severe neurological signs and potential death (thiamin deficiency) (Atkinson 1996; Aulerich et al. 1999).

Mink and Toxicology:

The mink, as an indicator species of environmental contaminant exposure and ecosystem health, is approved by the U.S. Environmental Protection Agency (U.S.

EPA) and recognized by the National Academy of Science as a preferred toxicological model (Calabrese et al. 1992; Aulerich et al. 1999). A ranch-raised strain of American mink represent a wildlife species that occupies a high trophic level and consumes prey affected by environmental contaminants, such as PCBs. In addition, the mink can be an important mammalian model for wildlife toxicity studies because of its presence in threatened habitats, toxicity studies can be done in a controlled laboratory situation, and there exists a common body of knowledge on mink husbandry and biology (Joergensen 1985; Calabrese et al. 1992; Lipscomb et al. 1996; Aulerich et al. 1999).

Mink are among the most sensitive mammals to environmental contaminants, especially PCBs (Aulerich and Ringer, 1977; Ringer et al. 1981; Aulerich et al. 1985, 1987). Studies have shown a wide range of sensitivities to commercial PCB mixtures and to the limited number of individual PCB congeners that have been investigated (2,2',3,3',6,6'- hexachlorobiphenyl [PCB 136], 2,2',4,4',5,5'-hexachlorobiphenyl [PCB 153], 3,3',4,4',5,5'-hexachlorobiphenyl [PCB 169], and TCDD (Chapter 2; Aulerich et al. 1985, 1987; Hochstein et al. 1988; Patnode and Curtis, 1994). Reproductive functions in mink are often disrupted as a result of exposure to PCBs (mixtures and congeners) due to the sensitivity of mink to such compounds (Aulerich and Ringer 1977; Aulerich et al. 1985, 1987; Backlin and Bergman, 1992; Kihlstrom et al. 1992).

Reproduction:

The mink is defined as a seasonal breeder. The annual breeding season is regulated by the length of day (photoperiod) (Aulerich et al. 1999). Unlike some of the other members of this order, mink are short-day breeders, meaning that the shortening

of day length triggers the reproductive season (Boissin-Aggase et al. 1986; Aulerich et al. 1999). The female mink has induced ovulation and delayed implantation, which are characteristics shared by other members of the family Mustelidae. Ovulation is induced by copulation, and it is believed to induce the release of luteinizing hormone from the pituitary gland, which then acts on mature follicles to induce their rupture (Murphy 1996). Delayed implantation is also known as embryonic diapause, which is a variable period in the mink terminated by implantation. Implantation follows the initial increase in progesterone by five to 10 days, and is influenced by the photoperiod, where an increase in photoperiod decreases the length of embryonic diapause (Murphy 1996; Aulerich et al. 1999). The mink also possesses unique reproductive characteristics, which include an abbreviated mating season and a curious pattern of repetition of ovulation in the presence of fertilized embryos (Murphy 1996).

In the male mink, as in other mustelids, testes size and spermatogenic activity varies during the year, and is positively correlated with elevated circulating testosterone concentrations (Onderka 1996; Aulerich et al. 1999; Wolf et al, 2000). The onset, as well as the regression, of testicular development in mink is triggered by an increase in testosterone beginning in October-November, and another increase in late March to mid-April (Aulerich et al. 1999; Wolf et al. 2000). This cycle is mediated through the photoperiod, resulting in the testes descending only during the breeding season in March (Aulerich et al. 1999). The mink penis contains a penile bone called the os penis or baculum. This feature is shared with other species of mammals, such as the walrus (*Odobenus rosmarus*) and rodents.

C. **Mink Skeletal System** (Axial Skeleton):

Skull:

The skull is part of the axial division of the skeleton. The skull is comprised of several bones fused together by sutures, part of which compose the neurocranium. The neurocranium forms a rigid, protective enclosure for the brain and sensory organs (eyes, nose, and ears) (Radke and Chiassom, 1998; Smith and Schenk, 2000).

The anterior of the skull, called the splanchnocranium, is comprised of facial bones that support the digestive and respiratory systems (Radke and Chiassom, 1998). The general bones appropriate for this study include the nasal bones, palatines, the premaxilla (or incisive bones), and specifically the maxillae and the mandibles. The premaxillae contain the sockets for the incisor teeth and articulate caudally with the maxillary bones. The maxillae are large paired dentary bones that are caudal to the premaxilla and have sockets for the upper canines, premolars, and molars. The mandibles are a pair of fused dentary bones that articulate with each other in the rostral midline by the mandibular symphysis. The mandible also has sockets for all of the lower teeth are in the portion of the mandible called the body (Radke and Chiassom, 1998).

Periodontium (Teeth):

Mink are true predators, therefore requiring specialized dentition to shear tough tissue, such as flesh, tendons, cartilage, and bone. The term palatal dentition is used to refer to teeth in the upper jaw, and lingual is used for teeth in the lower jaw (Radke and Chiasson, 1998). Kits are born without teeth, but ridges for future tooth eruption are

present (Hunter and Barker, 1996). Mink have both deciduous teeth and permanent teeth, with permanent teeth eruption being complete by approximately 2.5 months of age (Aulerich and Swindler, 1968*a*, 1968*b*). As with most mammals, there are four types of teeth that make up the permanent dentition of the mink: incisors, canines, premolars, and molars (Radke and Chiasson, 1998; Aulerich and Swindler, 1968*a*, 1968*b*).

The Tritubercular Theory in dental nomenclature was used. The number of teeth presented in the numerator, for example, is for a single quadrant. Incisor (I), canines (C), premolar teeth (PM), and molar teeth (M) are represented in each quadrant of the mink jaw for a total of 34 permanent teeth as designated by the formula. Superscript designates teeth located in the maxilla, subscript denotes teeth in the mandible.

Figure 1-3. The dental formula for the permanent dentition of mink (Aulerich and Swindler, 1968*a*, 1968*b*).

$$\begin{array}{lcccc} \text{Maxilla} & 3 & 1 & 3 & 1 \\ & \mathbf{I}\text{—}; & \mathbf{C}\text{—}; & \mathbf{PM}\text{—}; & \mathbf{M}\text{—} \\ \text{Mandible} & 3 & 1 & 3 & 2 \end{array} = 34$$

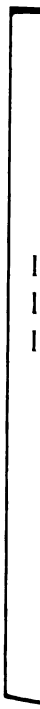


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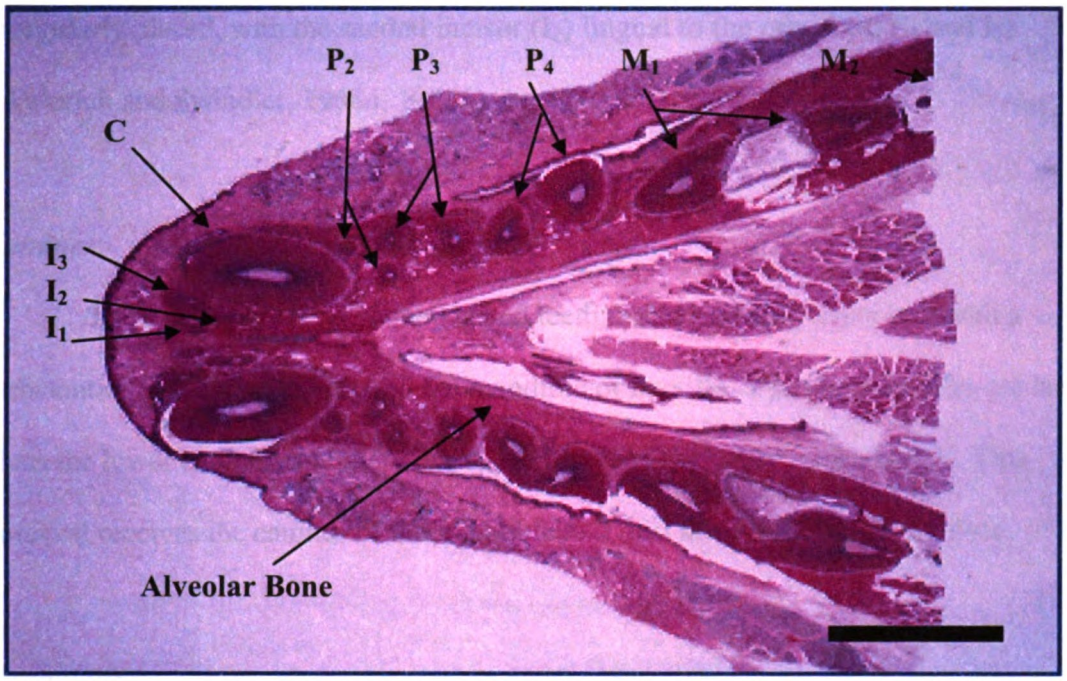
Figure 1-4. Sub-gross photomicrograph depicting normal permanent mandibular dentition of a standard dark mink (*Mustela vison*). H & E.



* The teeth are differentiated into four categories: Incisors (I)3/3; Canines (C)1/1; Premolars (P)3/3; and Molars (M)1/2. Lower incisors are irregularly placed in the mandible with the I_2 situated lingual to I_1 and I_3. The premolars have two roots per tooth. The mandible has two molars, with M_2 being a single-rooted tooth. H & E. Bar = 5 mm.

Incisors:

The six incisors of each jaw are simple teeth, each with a single cusp and root. The permanent incisors are larger, and erupt lingual to the deciduous incisors. Size does vary noticeably, with the third incisor (I^3) being largest of the three in the upper jaw. The incisors of the premaxilla are set transversely. In the mandible, the incisors are irregularly placed, with the second incisor (I_2) lingual to the other two (I_1 and I_3) (Aulerich and Swindler, 1968*a*, 1968*b*).

Canines:

The canines are large, cone-shaped teeth that are slightly recurved, with a substantial root anchoring then to the mandible or maxilla. The upper canines are larger than the lower canines, and there is a space between the upper canine and I^3. This interval receives the canines of the mandible and thus allows the mouth to close.

Premolars:

There are three upper and three lower premolars, and the deciduous and permanent premolars are very similar. The premolars in general are more complex in their structure, as they are sectorial in nature (Aulerich and Swindler, 1968*a*, 1968*b*). The large cusps of the premolars meet to form the carnassials, which are used to shear tissues (Radke and Chiasson, 1998).

Molars:

The dentition of the mink reflects that the species is a true predator and specialized flesh-eating diet. Therefore, there has been a reduction in the post-carnassial molars because grinding of plants is not required for the diet (Aulerich and Swindler, 1968*a*; Radke and Chiasson, 1998). The unique buccolingual dimension of the first upper molar of the mink and other Mustelidae is a characteristic that distinguishes this subfamily from all other carnivores (Aulerich and Swindler, 1968*a*).

Gingiva:

A complex relationship is necessary to maintain a healthy periodontium that supports adequate function of teeth (Jones and Boyde, 1999). This includes four primary components, periodontal ligament, alveolar bone (lamina dura), the gingiva, and cementum (Bartold et al. 2000). The periodontal ligament supports the tooth, and through these fibers functioning teeth are linked to each other, the gingiva, and the alveolar bone (Jones and Boyde, 1999). Normal gingiva covers the alveolar bone and tooth root to a level just coronal to cementoenamel junction (Bartold et al. 2000). The gingiva is comprised of two tissue types, epithelial tissue (overlying tissues) and connective tissues (fibrous underlying tissues) (Bartold et al. 2000). Both tissue types are responsible for regulation of normal function, repair or regeneration following injury, and/or protection associated with inflammatory responses (Bartold et al. 2000).

D. Oral Diseases:

Description of oral diseases described in mink are relatively limited. The most common oral "disease" in mink is the lodging of foreign objects, such as fish or chicken bones. If sharp fragments penetrate the oral cavity, it causing local abscesses behind the teeth, under the tongue, or may even penetrate the esophagus, leading to severe infection (Hunter and Barker, 1996).

Inflammatory responses have been reported as tooth abscesses and gingivitis affecting older mink. Cellulitis is seen in mink kits with erupting teeth and is associated with bacteria (Hunter and Barker, 1996). In addition, congenital abnormalities have been reported for the oral cavity in the mink. These include cleft palate, bradygnathia (abnormal shortening of the mandible) and prognathia (abnormal protrusion of the mandible) (Hunter and Barker, 1996). Older ranch mink have been observed with a heavy plaque load, but no confirmed periodontitis has been reported.

Periodontal Disease:

Periodontitis is a very common chronic inflammatory disease in humans and some animal species. The manifestation of periodontitis requires the presence of oral bacterial flora and the host inflammatory response, as well as environmental factors (Bartold et al. 2000). Studies now suggest that changes in local bacterial density and species composition may contribute to periodontal diseases, i.e. nearly 500 bacterial strains have been identified in the subgingival crevice of humans (Kroes et al. 1999). Calculus build-up results from an accumulation of bacteria on the tooth surfaces

adjacent to the supragingival and subgingival tissues and contributes to the recession of the gingiva and the initiation of the inflammatory response (Jeffcoat 1999; Bartold et al. 2000). The response then spreads to the tooth socket and deeper into the periodontal tissues, until the alveolar bone is lost. At this stage the lesion is termed periodontitis. Destruction of periodontal tissues including periodontal ligament and alveolar bone, is followed by subsequent tooth loss.

Neoplasia:

Neoplasms are differentiated by morphologic and behavioral characteristics. Benign tumors are non-cancerous, and are generally localized, slow growing, composed of cells that are well differentiated, do not invade surrounding tissue, have limited potential for growth and do not metastasize to distant sites. Criteria for malignant neoplasia include higher proliferative activity, invasiveness of atypical cell type and diffuse infiltration, and tissue destruction. Malignant neoplasms tend toward rapid growth, may contain anaplastic cells, and can metastasize to other organ sites. Malignant neoplasms tend to be more aggressive and characteristically invade surrounding tissue, therefore are often more osteolytic than benign neoplasia (Myer 1994; Thompson and Pool, 2002).

Types of Neoplasms:

Various proliferative lesions of the oral cavity (especially in canines) have been reported that included epulides, malignant melanomas, fibrosarcomas, osteosarcomas,

squamous odontogenic tumor, pseudo-carcinomatous hyperplasia, and squamous cell carcinoma (SCC) (Berg 1998; Yoshida et al. 1999*b*).

Epulides are localized tumor-like lesions of periodontal origin, which can be categorized as both non-neoplastic reactive and neoplastic lesions (Yoshida et al. 1999*a*). Epulis have also been described as a non-specific, localized growth of the gingiva (Gardner 1996). Some acanthomatous epulides have exhibited bone invasion characteristics that resemble SCC (Yoshida et al. 1999*b*). Based on the literature, acanthomatous epulides, peripheral ameloblastoma and basal cell carcinoma of the gingiva are most likely the same lesion (Gardner 1996).

The squamous odontogenic tumor (SOT) is a benign lesion of the periodontium that arises from the rest of Malassez in the periodontal tissue. Therefore, the SOT is most likely not from oral squamous epithelium (Tatemoto et al. 1989).

Pseudo-carcinomatous hyperplasia can be caused by an inflammatory process that originated in the dental lamina in a mandibular osteitis case reported by Penneau et al. (1980). Pseudoepitheliomatous hyperplasia, may be a similar diagnostic lesion to pseudo-carcinomatous hyperplasia. Histopathologically, pseudoepitheliomatous hyperplasia is an inflammatory lesion in juveniles that can become highly proliferative and assume neoplasm-like characteristics (Bill et al. 2001). Irregular infiltration and extension of atypical cells into the submucosa is uneven, accompanied by the development of jagged epidermal cell masses, leading to the development of SCC, and demonstrate no evidence of metastasis (Bill et al. 2001). Thus, SCC in adolescents can present itself as pseudoepitheliomatous hyperplasia that initiated as an ulcerative lesion

with no palpable nodules, and radiographic examination revealed erosion of the alveolar bone (Bill et al. 2001).

Squamous cell carcinoma of the oral cavity in cats and dogs is an aggressive disease, which frequently invades the periosteum of the underlying bone in up to 77% of canine cases. Often the early lesions of gingival squamous cell carcinoma are relatively small nodular masses along the dental arcade. These nodules do not always form fleshy masses, and rarely metastasize to distal sites, often at a rate of less than 3%. Lymphatic drainage from the gums is more restricted and therefore limits potential for metastases (Konde 1994; Thompson and Pool, 2002). Frequently, lesions referred to in the literature may have been reported elsewhere under a different lesion or name, but truly being the same diagnosis.

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CHAPTER 2

Female Mink (*Mustela vison*) Fed Diets Containing 3,3',4,4',5-Pentachlorobiphenyl (PCB 126). I: Reproductive Performance and Kit Survivability

Female Mink (*Mustela vison*) Fed Diets Containing 3,3',4,4',5-Pentachlorobiphenyl (PCB 126). I: Reproductive Performance and Kit Survivability

ABSTRACT

This study was conducted to determine if dietary exposure to 3,3',4,4',5-pentachlorobiphenyl (PCB 126) would affect reproductive success of female mink (*Mustela vison*). Standard dark, female mink were fed diets containing PCB 126 at concentrations of 0, 0.24, 2.4, and 24.0 μg PCB 126/kg feed from 21 days prior to breeding until weaning of their kits at six weeks of age. There were no significant differences in the number of females that whelped or the average litter size between dams that were fed the diet containing 0.24 μg PCB 126/kg feed and the control group. In addition, kit body weights at birth, three weeks and six weeks, and kit survivability through weaning (six weeks of age) were also similar between these two groups. Females fed 2.4 and 24.0 μg PCB 126/kg feed had confirmed matings, but failed to whelp. Histological examination of their uterine horns verified fetal implantation sites, or placental scars indicating partial embryo development. These results demonstrate that PCB 126 causes severe reproductive dysfunction in the mink at dietary concentrations as low as 2.4 μg PCB 126/kg feed.

INTRODUCTION

Polychlorinated biphenyls (PCBs), polychlorinated dibenzodioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) are ubiquitous environmental contaminants that are resistant to biological and chemical degradation. The toxicity of these contaminants can give rise to serious and significant effects on animal and human health. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is recognized as the most toxic congener of these chemicals (Van den Berg et al. 1998). The structurally similar, non-*ortho* substituted 3,3',4,4',5-pentachlorobiphenyl (PCB 126) is considered to be the most toxic PCB congener (Van den Berg et al. 1998). PCB 126 assumes a coplanar configuration that elicits toxic responses similar to those reported for TCDD, and PCB 126 is known to be prevalent in the environment (Safe 1990; Peterson et al. 1993).

Mink have been shown to be highly sensitive to PCB and TCDD exposure, which has resulted in disrupted reproductive function at relatively low concentrations (Aulerich and Ringer 1977; Aulerich et al. 1987; Hochstein et al. 1988). Reproductive dysfunction has been demonstrated in mink that have been exposed to commercial PCB mixtures, as well as PCB 'cocktails' comprised of selected PCB congeners (Aulerich and Ringer, 1977; Aulerich 1985; Backlin and Bergman, 1992; Kihlstrom et al. 1992; Jones et al. 1997). However, exposure to PCB mixtures has precluded the ability to ascertain specific PCB congeners associated with the observed adverse effects. Mink reproductive studies assessing the effects of single PCB congeners are limited. Two *ortho*-substituted congeners, 2,2',3,3',6,6'- hexachlorobiphenyl (PCB 136) and 2,2',4,4',5,5'-hexachlorobiphenyl (PCB 153), had no effect on reproduction in exposed

mink (Aulerich et al. 1985). Only one non-*ortho* PCB congener, 3,3',4,4'5,5'-hexachlorobiphenyl (PCB 169), has been investigated in regard to its toxic effects on reproduction in mink (Aulerich et al. 1985, 1987; Patnode and Curtis, 1994). Mink fed diets containing 0.01 mg PCB 169/kg feed had average litter size and kit survivability that was similar to controls. However, when mink were fed a diet containing 0.05 mg PCB 169/kg feed, 50% of the females died prior to whelping.

The toxic equivalency factor (TEF) is an estimate of the toxicity of a compound relative to TCDD based on individual toxicity reference values (TRVs). These TRVs are derived from *in vivo* studies that assess a number of endpoints (body weight loss, thymic atrophy, and endocrine dysruption) that are suggested to be mediated by the aryl hydrocarbon receptor (AhR), or from in vitro assays that utilize aryl hydrocarbon hydrolase [AHH] induction as an endpoint (Safe 1990; Giesy and Kannan 1998; Van den Berg et al. 1998). The individual TRVs can be highly variable; thus a TEF value for a particular congener may not accurately reflect the magnitude of its effects on a specific endpoint in a specific species.

The congener PCB 126 is considered to be a highly potent PCB congener, and has been assigned a mammalian TEF value of 0.1 (Van den Berg et al. 1998). Yet limited data are available on its effects in animals, and no information exists on the effects of this congener on reproductive function in mink. Thus, this study was designed to assess reproductive function in female mink fed diets containing PCB 126 and subsequent survivability of their offspring.

METHODS

Twenty-eight standard dark, primiparous, female mink were randomly selected from the Michigan State University (MSU) Experimental Fur Farm herd, weighed, and assigned to treatment groups so that each group had a similar average body weight. Siblings were not placed in the same group to minimize sensitivity to treatment attributable to genetic pre-disposition. All mink were housed individually in wire mesh breeder cages (61 cm L x 76 cm W x 46 cm H) with a wooden nest boxes (30 cm L x 22.5 cm W x 25 cm H) bedded with aspen shavings that attached to the outside of the cages. These cages were located within the MSU Experimental Fur Farm containment facility. Lighting in the room simulated the natural light/dark cycle for the Eastern Standard Time zone. Mink were started on their designated treatment diets on 05 February 2001, after a one-week acclimation period. Mink were observed daily for the duration of the study for any signs of toxicity including refusal to eat, changes in physical appearance, and behavior alterations.

Diet:

A standard ranch diet (Table 2-1), which was supplemented with 0.24, 2.4, or 24.0 μg PCB 126/kg feed, was used throughout the study. The standard ranch diet without supplemental PCB 126 was used as the control diet. A PCB 126 premix was prepared by first dissolving 5000 μg of the congener (Ultra Scientific, North Kingstown RI; 99% purity) in 2 mL hexane. The appropriate quantity of PCB 126 was then added to 80 mL corn oil and mixed well. The corn oil containing PCB 126 was then added to

1 kg dry mink cereal, followed by thorough mixing. The PCB 126-cereal premix was added to the remaining ingredients of the standard diet (Table 2-1) and thoroughly mixed. Samples of each diet were collected for PCB analysis in I-Chem® jars (I-Chem, New Castle, DE). Additionally, samples of each diet for nutrient analysis were collected in Whirl-Pak® bags (Nasco, Fort Atkinson, WI). All feed samples were stored in an ultra-cold freezer (-74°C) until analysis. Diets were then packed in 2-liter plastic containers and stored in a walk-in freezer (-7°C). As feed was needed, containers were transferred to a walk-in cooler (4°C) for thawing. Each two-liter container provided a two-day supply of feed. During the maintenance period and breeding season, all mink were fed to condition them for optimum reproduction. Water was available *ad libitum*.

Reproduction:

Breeding of females began during the third week of February, which was approximately one week earlier than usual for mink housed in open-sided sheds, because the females showed signs of estrus by pronounced vulvar swelling. All females were bred according to the MSU Experimental Fur Farm standard operating procedures. Non-exposed males were used for breeding. Mating was attempted once early in the 21-day breeding period, and then again eight days later. All potential matings were verified by the presence of normal motile spermatozoa in the postcoital vaginal aspirations collected immediately after mating. An initial positive mating was followed by a second breeding attempt on the following day with a different male, as well as another attempt on the eighth and ninth days after the first successful mating. Females not having a verified mating were designated as non-breeders.

Table 2-1. Composition and nutrient analysis of the basal diet used for the PCB 126 experiments.

Feed Composition	**%**
Ingredients	
Mink cereal [a]	30.00
Duck by-products [b]	32.00
Water	21.00
Menhaden fish meal [c]	7.00
Eggs (spray-dried) [d]	2.00
Liver (freeze-dried) [e]	2.00
Corn oil	1.00
Phosphoric acid (85%) [f]	0.80
Vitamin premix [g]	1.5 %
d-Biotin/kg [h]	0.07 mg
Larvadex [i] 2SL	62.0 mL
Proximate Analysis [j] (on a dry weight basis)	**%**
Moisture	45.65
Fat	24.16
Protein	35.02
Crude fiber	2.79
Ash	9.74
Calcium	2.03
Phosphorus	1.78
Potassium	0.86
Magnesium	0.19
Sodium	0.65
Total digestible nutrients (*TDN*)	96.90
Iron	367 ppm
Manganese	47 ppm
Copper	7 ppm
Zinc	96 ppm

[a] XK-40 mink cereal, XK Mink Foods, Inc., Plymouth, WI
[b] United Feeds Inc., Plymouth, WI
[c] Omega Protein, Inc., Hammond, LA
[d] Van Elderen, Inc., Martin, MI
[e] Van Elderen, Inc., Martin, MI
[f] Alexander Chemical Corp., Kingsbury, IN
[g] MSU Vitamin PMX, Akey, Lewisburg, OH
[h] Archer Daniel Midland, Des Moines, IA
[i] Larvadex ® 2SL, Novartis, Greensboro, NC
[j] Litchfield Analytical Services, Litchfield, MI

Females were checked daily during the whelping period (mid-April to mid-May) for evidence of whelping. Gestation length was defined as the number of days from the last successful mating to parturition, a period that encompasses a variable period of delayed implantation (diapause) as well as true pregnancy. Livebirths and stillborns were counted, sexed and weighed on the day of birth. Kits, as well as their dams, were weighed again at three and six weeks of age. Kits were weaned at six weeks of age (mid-June) and maintained on their respective treatment diets.

Necropsies:

In May 2001, a sub-group of nine non-whelping females was euthanized using CO_2 to determine reproductive status. These females included two control females, three females from the 2.4 μg PCB 126/kg feed group, and four females from the 24.0 μg PCB 126/kg feed group. A gross examination was performed on each dam and reproductive tissues were collected and fixed in 10% buffered formalin (pH 7.4) for subsequent histopathological assessment using hematoxylin and eosin-stained sections. Uteri were grossly and histologically examined for implantation sites, as well as for signs of early embryonic death or late fetal death. In addition, ovaries were histologically examined for signs of ovulation and degree of follicular development. All female mink that died on trial were necropsied as described above.

Statistical Analysis:

Data were analyzed using SAS® software (Statistical Analysis Systems, version 8.0, Cary, NC) by analysis of variance, and least square means of treatment parameters.

The mixed model with repeated measures was used, and data were log transformed to meet the assumption of the mixed model for kit body weights. Residual plots were used to determine normal probability distribution and to check outlier status. The Tukey's method was used for adjustment of *p*-value in multiple tests. The Chi-square test was used to analyze kit survival. The level of significance was based on a Type I error rate of $\alpha = 0.05$ ($p \leq 0.05$).

RESULTS

Reproduction:

The number of females with confirmed matings per group was similar (Table 2-2). The average number of confirmed matings per group ranged from 1.28 to 1.71. Only females from the control group and the 0.24 μg PCB 126/kg feed group whelped, also noting that all females with confirmed matings in these two groups whelped (Table 2-2). The length of gestation between the control group and the 0.24 μg PCB126/kg feed group did not differ significantly (Table 2-2) and ranged from 46 to 55 days and 46 to 59 days for the control and 0.24 μg PCB 126/kg feed groups, respectively.

Of the nine non-whelping females that were examined, one control female, two females from the 2.4 μg PCB 126/kg feed group and one female from the 24.0 μg PCB 126/kg feed group did not have evidence of placental scars. The remaining females had implantation sites located throughout the uteri (Figure 2-1), indicating fetal death in the uterine horns. In addition, ovaries collected from non-whelping females from the 2.4 and 24.0 μg PCB 126/kg feed groups contained corpora lutea in different stages of

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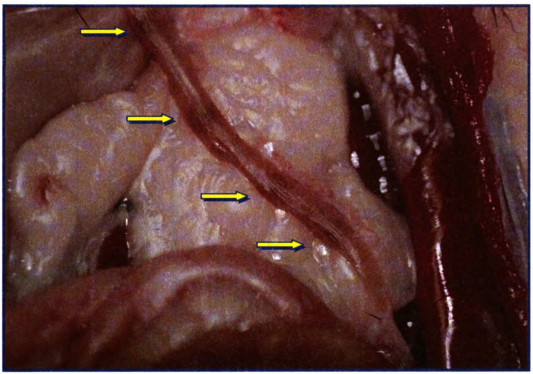
involution, indicating active ovaries. Changes in mucosal thickness of uterine horns were also apparent in females from the 2.4 and 24.0 μg PCB 126/kg feed groups. Additionally, the presence of hemosiderin-laden macrophages in the mucosal stroma was noted in one female from the 2.4 μg PCB 126/kg feed group.

There were no significant differences in the total number of kits whelped per litter (livebirths plus stillborns) between the control and the 0.24 μg PCB 126/kg feed groups (Table 2-3). Of the whelped kits, 28.6% (control) and 33% (0.24 μg PCB 126 /kg diet) were stillborn or died within the first 24 hours postpartum. The average number of liveborn kits per whelping female was not significantly different with 3.75 and 4.0 livebirths per female for the control and 0.24 μg PCB 126/kg feed groups, respectively. Survivability and growth of kits whelped by females exposed to 0.24 μg PCB 126 /kg feed were not adversely affected when compared to controls through weaning or at 28 weeks of age (Tables 2-3 and 2-4).

Clinical Signs of Toxicity:

Animals exposed to the two greater concentrations of PCB 126 exhibited mortality as well as showed clinical signs of toxicity, including hind-quarter paralysis, anorexia, "wasting syndrome", poor coat condition, increased shedding with possible alopecia, eye infections and nasal discharge, bloody and tarry feces. Three females died after 42, 47 and 74 days of consuming the diet containing 24.0 μg PCB 126 /kg feed. In addition, one control female, one female in the 0.24 μg PCB 126 /kg feed group, and two females in the 24.0 μg PCB 126 /kg feed group died from non-treatment related

Figure 2-1. Implantation sites that were seen within a uterine horn of a bred female receiving the diet that contained 24.0 μg PCB 126 /kg feed, that did not whelp.



* Arrows (4) depict implantation sites at states of resorption.

Table 2-2. Reproductive performance of female mink fed diets containing PCB 126 at a dietary concentration of 0, 0.24, 2.4, or 24.0 µg PCB 126/kg feed.

PCB 126 Concentration (µg/kg feed)	# Female Bred/Total # Female	Confirmed Matings / Female*	# Female Whelped/ # Mated	Average Gestation Length (d) [1]
0	4/7	1.43 ± 0.61	4/4	50.75 ± 1.89
0.24	6/7	1.43 ± 0.39	6/6	51.67 ± 1.93
2.4	6/7	1.71 ± 0.42	0/6	---
24.0	5/7	1.29 ± 0.47	0/5	---

[1] Data presented as mean ± standard error (SE) of the mean.

Table 2-3. The effects of dietary PCB 126 on kit survivability from whelping through weaning at 6-weeks of age.

PCB 126 Concentration (µg/kg feed)	Average Litter Size (Livebirths)[1]	Average Stillborn[1]	*Number of Kits at Birth* Livebirths	Stillborn	Average Kit Survival at 3 Weeks[1]	Survivability (% Kits Weaned)
0	3.75 ± 0.91	1.5 ± 0.43	15	6	2.75 ± 0.37	55.95%
0.24	4.00 ± 0.74	2.0 ± 0.21	24	12	2.83 ± 0.34	56.83%
2.4	---	---	---	---	---	---
24.0	---	---	---	---	---	---

[1] Data presented as mean ± standard error (SE) of the mean.

Table 2-4. The effects of dietary PCB 126 on kit body weights at birth, and three and six and 28 weeks of age.

PCB 126 Concentration (µg/kg feed)	Kit Body Weights (g) [1, 2]				
	Birth	*3-Weeks*	*6-Weeks*	*28-Weeks*	
				Males	Females
0	8.63 ± 2.16	111.32 ± 16.96	241.78 ± 84.89	2106.00 ± 118.72	1296.75 ± 79.76
(n)	(15)	(11)	(9)	(4)	(4)
0.24	8.18 ± 2.34	96.52 ± 23.09	254.56 ± 55.59	1987.86 ± 125.75	1221.60 ± 67.37
(n)	(24)	(17)	(15)	(7)	(5)

[1] Data presented as mean ± standard error (SE) of the mean.
[2] Means with different superscripts in column are significantly different at $p \leq 0.05$.

causes. Squamous epithelial hyperplasia of the mandibles and maxillae was observed grossly in all of the females receiving the 2.4 and 24.0 μg PCB 126 /kg feed diet.

In general, kits whelped to dams in both the control group and the group receiving 0.24 μg PCB 126 /kg feed appeared normal, and few clinical manifestations of toxicity were observed. However, one litter in the 0.24 μg PCB 126 /kg feed group had general signs of unthriftiness and poor health, with a preliminary diagnosis of aplastic anemia or red cell aplasia. These are typically considered genetic disorders, and thus were considered to be non-treatment related.

DISCUSSION

The results of the present study demonstrate that PCB 126 adversely affects reproduction in mink. Dietary exposure to concentrations as low as 2.4 μg PCB 126/kg feed induced complete reproductive failure in mink. This concentration of PCB 126 is an order of magnitude greater than environmentally relevant concentrations with the toxic equivalent (TEQ) concentration being approximately three times greater than environmental TEQs being previously reported (Heaton et al. 1995; Tillitt et al. 1996; Bursian et al. 2003). In this study, the lowest observed adverse effect level (LOAEL) based on reproductive failure induced by PCB 126 was 2.4 μg /kg feed, while the no observed adverse effect level (NOAEL) was 0.24 μg PCB 126/kg feed.

The toxic equivalent method relates the toxicity of TCDD-like chemicals (specific PCDD, PCDF and PCB congeners) to the toxicity of TCDD. The TEF

constitutes an estimate of the toxicity of a compound relative to TCDD, and applies to aryl hydrocarbon receptor (Ah-R) mediated responses (Van den Berg et al. 1998). Each congener is assigned a TEF value, for instance, the TEF of PCB 126 is 0.1 (Van den Berg et al. 1998). Toxic equivalents are derived by multiplying the TEF of a particular congener by the concentration of the chemical in the sample of interest (Safe 1990; Van den Berg et al. 1998). In a sample containing a number of different PCDD, PCDF and PCB congeners, the total TCDD-like activity can be calculated by summing the TEQs contributed by each congener.

The TEQ concentration of the 0.24 μg PCB 126/kg feed diet was 24.0 ng TEQ/kg feed, while the dietary TEQs at the LOAEL was 240.0 ng TEQs/kg. Heaton et al. (1995) and Tillitt et al. (1996) reported that diets containing 10, 20, and 40 % Saginaw River fish contained 19.4, 40.0, and 80.8 ng/kg dietary TEQs, respectively. If the currently accepted TEF values reported by Van den Berg et al. (1998) are used for the above mentioned study, the corrected TEQ concentrations are 22.2, 43.0, and 85.0 ng/kg feed TEQs for the 10, 20, and 40 % Saginaw River fish, respectively (Heaton et al. 1995; Tillitt et al. 1996; Van den Berg et al. 1998).

In the present study, the estimated total PCB 126 consumption for an average 54-day exposure period was 19.1 μg PCB 126/kg BW (2.4 μg PCB 126/kg diet), and 232.6 μg PCB 126/kg BW (24.0 μg PCB 126/kg dietary exposure). Females that received the diet containing 0.24 μg PCB 126/kg feed had an average total PCB 126 consumption of 4.47 μg PCB 126/kg BW during their 133 days of exposure for dams rearing litters through weaning.

The TEQ concentration of the 0.24 μg PCB 126/kg feed diet for the NOAEL was 24.0 ng /kg feed. The LOAEL (2.4 μg PCB 126/kg feed) TEQ concentration was 240.0 ng/kg diet at which concentration complete reproductive failure was observed. In the Heaton et al. (1995)/Tillitt et al. (1996) study, the LOAEL, based on kit survivability, was the lowest PCB concentration fed (0.72 mg total PCB/kg diet) or 22.2 ng TEQs/kg feed using the TEF values presented in Van den Berg et al. (1998). Thus, the NOAEL TEQ concentration in the present study was equivalent to the LOAEL TEQ concentration in the Heaton et al. (1995)/Tillitt et al. (1996) study. It is of interest to point out that kit survivability at 24 ng TEQs/kg feed in the present study is quite comparable to kit survivability at 22.2 ng TEQs/kg feed in the Heaton et al. (1995)/Tillitt et al. (1996) study, but the relatively poor whelping and survivability in the control animals in the present study preclude statistical significance.

Other parameters of reproductive performance that were measured were comparable between the current study and Heaton et al. (1995) for the two exposure groups (24.0 ng TEQs/kg feed and 22.2 ng TEQs/kg feed, respectively). In fact, Heaton et al. (1995) reported a higher number of livebirths per litter, with 3.8 livebirths, as compared to 2.8 livebirths observed in the present study. Average litter size was smaller in the present study with an average of 4.0 kits per litter as compared to 5.3 kits per litter reported by Heaton et al. (1995), as well as birth weight (8.16 g) was lower in the present study as compared to 9.76 g from Heaton et al. (1995).

Litter size indicates the capability of the dam to carry fetuses to parturition. The number of kits whelped per litter was recorded within the first 24 hours of whelping. The number of living kits per mated female (3.75 and 4.00 kits for the control and 0.24

μg PCB 126/kg feed groups, respectively) was not influenced by exposure to 0.24 μg PCB 126 in the feed. Net natality, or the number of young weaned per female, was not significantly different between the control group at 2.25 and the mink exposed to 0.24 μg PCB 126/kg feed which weaned 2.83 kits per female.

Aulerich et al. (1985, 1987) reported 50% mortality in female mink following 88 days of dietary exposure to 0.05 mg PCB 169/kg feed, thus reproductive function could not be assessed. The TEF value of PCB 169 is 0.01 (Van den Berg et al. 1998). Therefore, the TEQ concentration at which 50% mortality in mink was reported was 500 ng TEQs/kg feed. The *ortho*-substituted congener PCB 136 had no reproductive effects at a dietary concentration of 2.5 mg PCB 136/kg feed (Aulerich et al. 1985).

Kit body weights reported by Heaton et al. (1995) were substantially greater, and kit productivity was higher in all groups except the "40% carp" group as compared to the present study. This may be explained by the greater than average length in gestation that was reported by Heaton et al. (1995) (64.5, 58.7, 55.2, and 58.7 days for control, 22.2, 43.0, and 85.0 ng TEQs/kg feed groups, respectively), which allowed the kits to develop to a greater extent prior to parturition. The average reported gestation length for standard dark, female mink in the MSU Fur Farm herd during the same time period, as the present study was 49.1 days. For single-mated dark mink, the average gestation period reported is 51.2 days (Bowness 1968). In the study by Heaton et al. (1995), the overall average length in gestation was 59.3 days.

Several studies have indicated that exposure to commercial PCB mixtures, environmentally-derived PCBs, and selected PCB congeners cause adverse reproductive impairment in mink although apparently ovulation, fertilization and implantation are

apparently not disrupted (Aulerich and Ringer, 1977; Aulerich et al. 1985; Backlin and Bergman, 1992; Jones et al. 1997). Mink are among the most sensitive species to the PCB congeners that are chemically similar to TCDD and mink are naturally exposed to many of these compounds in the environment via their diet (Aulerich et al 1985). The effects of TCDD exposure in mink have been investigated, but the effects on reproduction could not be clearly determined (Hochstein et al. 2001).

It is evident, based on histological examination of reproductive tracts, that PCB 126 at 2.4 and 24.0 μg PCB 126/kg feed did not prevent ovulation, fertilization and implantation in mated female mink. Females from both of these dietary groups had implantation sites located throughout both uterine horns, and ovaries were active and producing follicles. The implantation sites examined appeared as dark colored bands, also termed placental scars. These pigmented areas of uterine tissue are marking sites of previous placental attachment, which occur in species with deciduous placentae, including the family Mustelidae. These scars are interpreted as signs of resorption of conceptus, or early fetal stage death, and are commonly used indices that provide accurate determination and confirmation of pregnancy that can also be used to approximate litter size (Harder and Kirkpatrick, 1994).

The reproductive disruption induced by PCB 126 at the two higher doses (2.4 and 24.0 μg PCB 126/kg feed) may have been caused by PCB-mediated action on female reproductive hormones, or altered endocrine cycling as was observed in Backlin et al. (1997). Post-implantation embryo mortality may occur in female mink as a result of interference with hormones required for maintenance of pregnancy (Backlin et al. 1997). Earlier studies do indicate hormonal involvement as a mechanism by which PCB

exposure induces fetal death (Backlin et al 1997). The effects of PCBs and dioxins on the endocrine system are pronounced (Birnbaum 1994), including the reproductive dysfunction observed in mink exposed to PCBs. The endocrine system plays an essential role in the preparation and maintenance of the reproductive system for pregnancy. Estrogen is necessary for normal uterine development and maintenance, as well as for the continuation of pregnancy (Peterson et al. 1993). TCDD exposure caused a decrease in plasma concentrations of estrogen and progesterone in rhesus monkeys (Barsotti et al. 1979). Results from chronic dietary exposure to TCDD suggested suppression or inhibition of the estrous cycle in rats (Kociba et al. 1976). Impairment of progesterone receptor function has been observed *in uteri* of pregnant mink after a single dose of the coplanar PCB 169 (Patnode and Curtis, 1994; Backlin et al.1997).

Clinical signs of the animals that died on-test included wasting syndrome, adipose depletion, alopecia, ascites, and bloody and tarry feces. These signs were seen throughout all groups of adult females receiving PCB 126. The female mink receiving 24.0 μg PCB 126/kg feed also had notably thickened, deformed, and elongated toenails, which compares with other studies exposing mink to TCDD and PCB 126 (Hochstein et al. 1998; Render et al. 2000*a*, 2000*b*; Beckett et al. 2002). Clinical signs will be discussed in detail in Chapter 3. A lesion, initially identified as squamous epithelial proliferation of the mandibles and maxillae, was observed clinically in all of the females receiving the 2.4 and 24.0 μg PCB 126 /kg feed diet and will be discussed in detail in Chapter 4. One female that died on-test from the control group became agalactic, as well as had uroliths, two factors that contributed to the decrease in growth and survival of her five kits.

CONCLUSIONS

In conclusion, dietary administration of PCB 126 produced complete reproductive failure in mink at concentrations of 2.4 and 24.0 μg PCB 126/kg feed. Ovulation, fertilization and implantation in mated female mink did not appear to be altered by PCB 126 exposure. Females from both of these dietary groups had placental scars where implantations had failed. These placental scars were located throughout both uterine horns, and ovaries were active in these females. Based on the results from the current study, PCB 126 is highly toxic to mink, and caused severe impairment of reproductive function. Therefore, potential concentrations of PCB 126 in the environment may be high enough in certain areas to cause adverse effects in wild mink and other wildlife populations.

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CHAPTER 3

Adult Female and Juvenile Mink (*Mustela vison*) Fed Diets Containing 3,3',4,4',5-Pentachlorobiphenyl (PCB 126). II: Clinical, Hematological and Serum Chemistry Parameters

Adult Female and Juvenile Mink (*Mustela vison*) Fed Diets Containing 3,3',4,4',5-Pentachlorobiphenyl (PCB 126). II: Clinical, Hematological and Serum Chemistry Parameters

ABSTRACT

Polychlorinated biphenyls (PCBs) are ubiquitous environmental contaminants, and wildlife species are potentially exposed to these contaminants through their prey items. In addition, PCBs and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) are known to cause adverse effects in many species. The purpose of the current study was to determine the biological effects, as assessed by clinical, hematological, and serum chemistry parameters, in adult female and seven-month-old mink (*Mustela vison*) caused by the dietary consumption of 3,3',4,4',5-pentachlorobiphenyl (PCB 126). Standard dark, female mink were fed diets containing PCB 126 at concentrations of 0, 0.24, 2.4, and 24.0 μg PCB 126/kg feed three weeks prior to breeding and continuing post-weaning of kits. Kits whelped to females fed diets containing PCB 126 were continuously exposed *in utero* and through lactation via the dam, and then by consuming the diet containing the same concentration of PCB 126 as their dam. Clinical signs induced by PCB 126 exposure in the females included bloody and black tarry feces, poor coat condition, eye infections, nasal discharge and wasting syndrome. Excessive toenail growth was observed in the front feet of the adult females. There was a significant decrease in mean absolute heart weight in the adult females fed diets containing 24.0 μg PCB 126/kg feed. Mean liver weight was significantly increased in the adult females receiving 24.0 μg PCB 126/kg feed as compared to controls. Significant decreases in hematological parameters were observed as RBC, HGB, HCT

and eosinophils in adult females. Increases in neutrophils, WBC and MCV were observed in dams fed PCB 126. Juvenile mink fed diets containing PCB 126 exhibited significant decreases in RBC, HGB and HCT. Serum chemistry parameters that were significantly decreased in dams exposed to PCB 126 included albumin, calcium, total protein, and TCO_2 concentrations, and anion gap. A substantial trend in reduced cholesterol concentrations was observed in a dose-related manner in adult mink, although not statistically significant. Juvenile mink fed diets containing PCB 126 expressed significant decreases in alkaline phosphatase activity, and cholesterol and glucose concentrations. Potassium concentrations were significantly increased in juvenile mink exposed to PCB 126 via their diet. This study correlated clinical, hematological and serum chemistry parameters to evaluate the adverse effects of PCB 126 exposure in mink. Results indicate that PCB 126 induced adverse effects in clinical, hematological and serum chemistry parameters in mink.

INTRODUCTION

Polychlorinated aromatic chemicals like polychlorinated biphenyls (PCBs), polychlorinated dibenzodioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) are ubiquitous contaminants in the environment. These contaminants are resistant to biological and chemical degradation, and their toxicity and bioaccumulation give rise to serious and significant impacts on wildlife and human health. Polychlorinated aromatic chemicals are highly lipid soluble and partition into fatty tissues of the animals that

consume them, with the assumption that these fat stores or bioaccumulated reserves are passed to the next highest level in the food web (Borlakoqlu and Haegele, 1991).

The individual congeners that comprise this group of polychlorinated aromatic chemicals can differ markedly in relation to their toxicity to a species, as well as the response(s) elicited from specific species. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is recognized as the most potent congener of this class of chemicals. The structurally similar 3,3',4,4',5-pentachlorobiphenyl (PCB 126), the PCB congener that has stereochemical and toxicological properties most similar to those of TCDD, is considered to be the most toxic PCB congener. It exhibits "TCDD-like" activities, and is prevalent in the environment (Safe 1990; Erickson 1997).

Mink are naturally exposed to many of these compounds in the environment via their diet. Being at the peak of the food web, a wide variety of prey items that mink incorporate as part of their diet have been exposed to environmental contaminants, and thus, mink are exposed to potentially bioaccumulated contamination through dietary sources. Mink are among the most sensitive mammalian species known to chemical compounds such as PCBs and TCDD (Aulerich and Ringer, 1977; Aulerich et al. 1987; Hochstein et al. 1988). Thus, mink are an important model species that can be used as a biological indicator of environmental contaminant exposure and ecosystem health.

Polychlorinated biphenyls are known to adversely affect basic biological functions and physiological homeostasis. A plethora of biological effects can occur in association with ingestion and uptake of PCBs, including "wasting syndrome", chloracne, edema, hepatic hypertrophy, porphyria, estrogenic activity, immunosuppression, and neoplasia (Bleavins et al. 1983; Aulerich et al. 1987; Safe

1990; Silberhorn et al. 1990; Hochstein et al. 2001). Biochemical, hematological, and morphological markers may help to characterize and assess exposure to PCBs, as well as to assess the health condition of the animal. In a chronic toxicity study, Hochstein et al. (2001) reported significant changes in hematology and biochemical parameters in mink fed diets containing 0.04 to 5.0 μg TCDD /kg feed. Edqvist et al. (1992) reported changes in biochemical blood parameters in mink as a result of exposure to commercial PCBs and individual or combinations of individual PCB congeners. The purpose of the current study was to determine the biological effects, as assessed by clinical, hematological, and serum chemistry parameters, in adult female and seven-month-old mink caused by the dietary consumption of PCB 126.

METHODS

This study was conducted at the Michigan State University (MSU) Experimental Fur Farm from February, 2001 through December, 2001. The methods used in this study are presented in detail in Chapter 2. However, they will be briefly described below.

Twenty-eight, standard dark, breeding female mink were randomly selected and assigned to one of four dietary treatments. All mink were housed individually at the MSU Experimental Fur Farm containment facility. Mink were started on their designated treatment diets on 05 February 2001, after a one-week acclimation period and 21 days prior to breeding. A standard ranch diet (Table 2-1) was used as the control

diet. For the treatment diets, the standard diet was supplemented with 0.24, 2.4, or 24.0 μg PCB126/kg feed. Water was available *ad libitum*. Mink were observed daily for the duration of the study for any signs of toxicity including, refusal or inability to eat (aphagia), changes in physical appearance, and behavior alterations (Table 3-1). The kits that were evaluated in this study were offspring that were whelped from dams exposed to 0.24 μg PCB 126/ kg feed (Chapter 2). No other females in the study were successful in whelping any kits.

Blood Collection:

Adult female mink were anesthetized with a 10:1 mixture of ketamine hydrochloride (25mg/mL; Ketaset®, Fort Dodge Animal Health, Fort Dodge, IA) and Xylazine-20 (20 mg/mL; Butler®, Ben Venue Laboratories, Inc., Bedford, OH) at 0.4 mL/kg BW that was administered via intramuscular injection. Approximately 5 mL of blood was collected from each mink via cardiac puncture using a sterile 5 mL syringe with an 18 gauge needle. Approximately 2 mL of blood were put in a clot tube for serum chemistry, and 2.5 mL of blood was put in an ethylenediaminetetraacidic acid (EDTA)-coated tube for hematology. Following collection, the blood samples were submitted to the MSU Clinical Pathology Laboratory for analyses.

Hematology parameters analyzed for each sample included red blood cell (RBC) count, white blood cell (WBC) count, hemoglobin (HGB) concentration, hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW), platelet volume (PLT) and leukocyte differential cell counts (neutrophils, lymphocytes, eosinophils, basophils, and

monocytes). Blood samples were analyzed using an Advia Model 120 analyzer (Bayer Corporation, Tarrytown, NY).

Serum biochemical analyses included concentrations of calcium (Ca), chloride (Cl), cholesterol (CHOL), creatinine (CREAT), albumin (ALB), total protein, total carbon dioxide (TCO_2), potassium (K), sodium (Na), glucose (GLUC), magnesium (Mg), serum iron (Fe), blood urea nitrogen (BUN), globulin (GLOB), anion gap (AG), and osmolality. Serum enzyme analyses included measurements of aspartate aminotransferase (AST), amylase (AMYL), alanine aminotransferase (ALT), alkaline phosphatase (ALK PHOS), creatinine kinase (CK), and sorbitol dehydrogenase (SDH) activities. Serum biochemical analyses were performed on an Abbott Spectrum Series II Analyzer (Abbott Laboratories, Irving, TX).

Necropsies:

Following blood collection, the adult females were euthanized using CO_2. A gross examination was performed prior to collecting tissues. Organs (adrenal glands, brain, heart, kidneys, liver, reproductive tract, spleen, and thyroids) were examined and weighed. A section of each tissue was then fixed in 10% buffered formalin (pH 7.4). Tissues were trimmed, embedded in paraffin, and then sectioned to 5 μm. Following this preparation, tissues were mounted on microscopic slides, and stained with hematoxylin and eosin for subsequent routine histopathological assessment.

Statistical Analysis:

Data were analyzed using SAS® software (Statistical Analysis Systems, version 8.0, Cary, NC) by one-way analysis of variance and least square means of treatment parameters. The mixed model procedure was used and the normality assumption was tested accordingly. Data were log transformed to meet the assumption of the mixed model. Residual plots were used to determine normal probability distribution and to check outlier status. The level of significance was based on a Type I error rate of 0.05 ($p \leq 0.05$). The Tukey's method was used for adjustment of p-value in multiple tests. Organ weights for the mink are presented as absolute weights (mean ± SE of the mean). No thyroid glands were collected from the adult females receiving 24.0 μg PCB 126/kg feed. Statistics for all parameter comparisons involving male and female kits were run in a sex by treatment comparison to determine any significant differences. If no differences were detected, males and females were grouped together by treatment, otherwise they are reported separately.

RESULTS

Mortality:

Three females on the diet containing 24.0 μg PCB 126 /kg feed died after 42, 47, and 74 days of being exposed to the PCB 126 diet, resulting in 29% mortality. One of these mink (24.0 μg PCB 126 /kg feed, 47-d) was euthanized due to hind-quarter paralysis in addition to decreased BW, it was also noted that the skull bones were brittle, and the front nails were splitting and also brittle. In addition, one control female

Table 3-1. Chart of daily observations for signs of toxicity in mink (*Mustela vison*) fed PCB 126 to monitor general health and physical condition.

Changes in Physical Condition:

- Weight changes
- Coat condition
- Stool characteristics
- Urination check
- Injury

Level of Activity:

- Increase
- Normal
- Decrease

Behavioral Alterations:

- Alertness
- 'Increased' aggression
- 'Increased' reclusion
- 'Increased/decreased' fearfulness
- Self-mutilation (fur and/or tail chewing)
- Feed wastage
- General uncleanliness

(264 days), one female from the 2.4 μg PCB 126 /kg feed group (128 days), and one female in the 24.0 μg PCB 126 /kg feed group (109 days) died from complications of uroliths in the kidney, which was considered non-treatment related.

Clinical Signs of Toxicity:

Clinical signs observed throughout all dietary groups receiving PCB 126 bloody and tarry feces, poor coat condition, eye infections and nasal discharge. Shedding was increased in PCB-126 exposed females as compared to control females. Noticeable shedding was first observed in the group of females receiving the 24.0 μg PCB 126 /kg feed diet. The patterns of shedding were not correlated with natural patterns of a seasonal molt.

Mink exposed to the two greater concentrations of PCB 126 showed signs of toxicity that included ascites (one mink, 24.0 μg PCB 126 /kg), anorexia and "wasting syndrome", resulting in depleted adipose tissue. Squamous epithelial proliferation of the mandibles and maxillae was clinically observed in all of the females receiving the 2.4 and 24.0 μg PCB 126 /kg feed diet.

As stated in Chapter 2, one litter whelped to a dam receiving the 0.24 μg PCB 126 /kg diet showed general signs of unthriftiness and poor health, with a preliminary diagnosis of aplastic anemia or possibly red cell aplasia. These are typically considered genetic disorders, and thus could be considered non-treatment related.

Toenails:

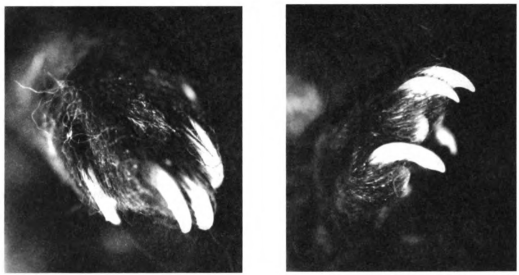
The adult female mink on PCB 126 diets had notably thickened, deformed, and elongated toenails (Figure 3-1). Increased nail growth was first noted in animals receiving the diet containing 24.0 μg PCB 126/kg feed within approximately five weeks of exposure. Nail growth was continuous and became pronounced in most mink in the two higher PCB 126 treatment groups after approximately 12 to 16 weeks of dietary exposure. Not all mink showed the same rate of growth, and growth rate was not measured in this study. Only the front nails had significant growth, as no unusual growth was seen in the toenails of the hind feet in any of the animals. Minimal changes in nail growth were noted in one juvenile male mink.

Necropsy Data:

The observed decrease in body weight (32%) of adult females exposed to 24.0 μg PCB 126/kg feed compared to controls was not significant (Table 3-2); although, this was considered a biologically significant decrease in body weight.

Heart weights were significantly decreased (24.3%) in dams in the 24.0 μg PCB 126 /kg feed group as compared to the control group (Table 3-2). Mean liver weight of the adult females in the 2.4 μg PCB 126 /kg feed group was significantly increased compared to the control group. No other significant changes in mean organ (adrenal glands, brain, kidneys, spleen, or thyroid glands) weights were observed in adult females. There were no significant differences in any organ weights for juvenile mink in this study (Table 3-3).

Figure 3-1. Increased toenail growth induced by dietary exposure to 24.0 μg PCB 126/kg feed in female mink 16 weeks into the trial was characterized by thickening, elongation, and deformity of the nails in the front feet. Nail growth in PCB 126-exposed mink was considered moderate and continued throughout the trial period, including the period while mink were on clean feed post-PCB 126 exposure.



Hematology:

Adult female mink fed diets containing PCB 126 had significant changes in hematological parameters (Table 3-4). Dams receiving the diet containing 24.0 μg PCB 126 /kg feed had significant decreases in RBC, HGB and HCT. Additionally, dams in this same dose group had significant changes in leukocyte differential counts. A significant increase in neutrophils, and a significant decrease in eosinophils and lymphocytes was observed in these adult females as compared to all other treatment groups. In addition to these changes, there were significant increases in WBC count and MCV in dams fed the 24.0 μg PCB 126 /kg diet. Although there was a trend-increase observed in platelet counts in the PCB 126-exposed adult females, these changes were not statistically significant. Juvenile mink in the PCB 126 treatment group (0.24 μg PCB 126 /kg feed) had significant decreases in RBC, HGB and HCT as compared to the control group (Table 3-6).

Serum Chemistries:

Several of the serum chemistry parameters in dams were significantly affected following exposure to PCB 126 (Table 3-5). A significant decrease in ALB was observed in the mink fed the diet containing 24.0 μg PCB 126 /kg feed compared to the other groups. Calcium and AG were significantly decreased in the 24.0 μg PCB 126 /kg group as compared to the 0.24 and 2.4 μg PCB 126 /kg feed group for Ca, and to the 0.24 μg PCB 126 /kg feed group for anion gap. Total CO_2 was significantly decreased in the 24.0 μg PCB 126 /kg group compared to the 2.4 μg PCB 126 /kg feed group, and total protein was significantly decreased in the 24.0 μg PCB 126 /kg group when

compared to the control group. Serum CHOL in the adult female mink was substantially reduced in a dose-related manner in all groups exposed to PCB 126, but it was not statistically significant (Table 3-5).

In the juvenile mink that consumed 0.24 μg PCB 126/kg feed, there were significant decreases in alkaline phosphatase activity, as well as CHOL and glucose concentrations as compared to the control group (Table 3-7). Potassium concentration was significantly increased in the juvenile mink exposed to 0.24 μg PCB 126 compared to control mink.

DISCUSSION

3,3'4,4'5-Pentachlorobiphenyl (PCB 126) is considered to be the most toxic of the PCB congeners. The results from this study and the previous reproductive study (Chapter 2) demonstrate the highly toxic potency of this PCB congener to mink. This supports previous reports that mink are among the most sensitive mammalian species to PCB toxicity. In the current study, mortality was reported in the treatment group receiving 24.0 μg PCB 126/kg feed, in which three females died during the course of the trial resulting in 29% mortality. However, based on the reported jaw lesion in this study (to be discussed in Chapter 4), there would have been 100% mortality if animals were allowed to continue on treatment. Hochstein et al. (2001) reported 16.7% mortality in mink exposed to a diet containing 5.0 μg TCDD/kg feed during a 125 day feeding

Table 3-2. Mean terminal body weight and mean organ weights of female mink fed diets containing 0, 0.24, 2.4, or 24.0 µg PCB 126/kg feed.

Parameter (g)	Dietary Concentration of PCB 126 (µg PCB 126/kg feed) [1, 2]			
	0 (n = 4)	0.24 (n = 7)	2.4 (n = 7)	24.0 (n = 4)
Body Weight	1154.25 ± 121.13	1117.43 ± 91.57	1159.14 ± 91.57	787.25 ± 121.13
Adrenal Glands	0.0935 ± 0.02	0.0838 ± 0.01	0.0779 ± 0.01	0.1281 ± 0.02
Brain	8.75 ± 0.36	8.36 ± 0.28	8.72 ± 0.28	8.86 ± 0.42
Heart	7.44 ± 0.45^{a}	6.46 ± 0.34^{ab}	7.03 ± 0.34^{ab}	5.63 ± 0.45^{b}
Liver	36.61 ± 5.62^{a}	40.13 ± 4.25^{a}	57.70 ± 4.25^{b}	42.55 ± 5.62^{ab}
Kidney	6.19 ± 0.68	7.32 ± 0.52	7.37 ± 0.52	6.68 ± 0.79
Spleen	3.47 ± 0.60	3.31 ± 0.45	4.75 ± 0.45	4.98 ± 0.60
Thyroid Glands	0.0588 ± 0.01	0.0581 ± 0.01	0.0666 ± 0.01	---

[1] Mean organ weights presented as absolute weight.
[2] Data are presented as means ± standard error (SE) of the mean.
Means in the same row with different letter superscript are significantly different ($p \leq 0.05$).

Table 3-3. Mean terminal body weight and mean organ weights of seven-month-old mink kits fed diets containing 0, 0.24, 2.4, or 24.0 µg PCB 126/kg feed.

	Dietary Concentration of PCB 126 (µg PCB 126/kg feed) [1, 2]			
	0 (n = 5)	0.24 (n = 11)	0 (n = 3)	0.24 (n = 3)
Parameter (g)	**Males**		**Females**	
Body Weight	2063.50 ± 122.79	1925.86 ± 92.82	1164.75 ± 173.65	1161.20 ± 109.83
Adrenal Glands	0.1355 ± 0.01	0.1141 ± 0.01	0.0793 ± 0.02	0.0674 ± 0.01
Brain	10.59 ± 0.30	10.27 ± 0.23	8.42 ±0.35	8.54 ± 0.27
Heart	10.16 ± 1.08	9.67 ± 0.75	6.69 ± 1.74	6.85 ± 1.34
Liver	72.37 ± 8.01	67.85 ± 5.60	40.33 ± 12.92	44.41 ± 9.99
Kidney	11.71 ± 1.06	9.59 ± 0.74	5.83 ± 1.53	5.73 ± 1.32
Spleen	5.04 ± 0.60	4.78 ± 0.42	3.64 ± 0.87	3.47 ± 0.75
Thyroid Glands	0.0729 ± 0.01	0.0593 ± 0.00	0.0484 ± 0.01	0.0417 ± 0.01

[1] Mean weights presented as absolute weight.
[2] Data are presented as means ± standard error (SE) of the mean.

Table 3-4. Effects of dietary PCB 126 exposure on hematological values in adult female mink.

Parameter	Unit	Dietary Concentration (µg PCB 126/kg feed) [1, 2] 0 (n = 4)	0.24 (n = 7)	2.4 (n = 7)	24.0 (n = 4)
White Blood Cell Count	x10.e3/µL	5.96 ± 1.35[a]	5.90 ± 1.10[a]	6.75 ± 1.02[a]	11.29 ± 1.35[b]
Red Blood Cell Count	x10.e6/µL	8.80 ± 0.36[a]	9.15 ± 0.30[a]	8.47 ± 0.27[a]	6.79 ± 0.36[b]
Hemoglobin	g/dL	16.65 ± 0.54[a]	16.93 ± 0.44[a]	16.23 ± 0.41[a]	13.73 ± 0.54[b]
Hematocrit	%	51.88 ± 1.98[a]	53.75 ± 1.62[a]	52.87 ± 1.50[a]	44.90 ± 1.98[b]
Mean Corpuscular Volume	fL	59.00 ± 1.47[a]	58.93 ± 1.20[a]	62.47 ± 1.11[ab]	66.33 ± 1.47[b]
Platelets		524.75 ± 93.41	693.83 ± 76.27	795.57 ± 70.61	812.50 ± 93.41
Neutrophils	cells/µL	39.53 ± 6.44[a]	40.03 ± 5.26[a]	45.60 ± 4.87[a]	68.23 ± 6.44[b]
Lymphocytes	cells/µL	52.53 ± 6.14[a]	54.00 ± 5.01[a]	48.99 ± 4.64[a]	25.83 ± 6.14[b]
Monocytes	cells/µL	2.65 ± 0.34	1.48 ± 0.28	2.24 ± 0.26	2.03 ± 0.34
Eosinophils	cells/µL	3.70 ± 0.66[a]	3.68 ± 0.54[a]	2.30 ± 0.50[ab]	0.53 ± 0.66[b]
Basophils	cells/µL	0.18 ± 0.03	0.13 ± 0.02	0.11 ± 0.02	0.13 ± 0.03

[1] Data are presented as means ± standard error (SE) of the mean.
[2] Means in the same row with different letter superscript are significantly different ($p \leq 0.05$).

Table 3-5. Effects of dietary PCB 126 exposure on serum chemistry parameters in adult female mink.

		Dietary Concentration (µg PCB 126/kg feed) [1, 2]			
Parameter	**Unit**	0 (n = 4)	0.24 (n = 7)	2.4 (n = 7)	24.0 (n = 4)
Sodium	mmol/L	152.25 ± 0.77	152.83 ± 0.63	152.43 ± 0.58	154.25 ± 0.77
Potassium	mmol/L	4.38 ± 0.21	4.37 ± 0.17	4.46 ± 0.16	4.63 ± 0.21
Chloride	mmol/L	115.00 ± 1.01[a]	114.00 ± 0.83[a]	113.86 ± 0.77[a]	120.50 ± 1.01[b]
TCO_2	mmol/L	23.75 ± 0.72[ab]	24.00 ± 0.59[ab]	25.29 ± 0.54[a]	22.75 ± 0.72[b]
Anion Gap	mmol/L	17.80 ± 0.75[ab]	19.20 ± 0.61[a]	17.74 ± 0.56[ab]	15.63 ± 0.77[b]
Total Protein	g/dL	6.23 ± 0.22[a]	5.92 ± 0.18[ab]	5.77 ± 0.16[ab]	5.20 ± 0.22[b]
Albumin	g/dL	3.10 ± 0.23[a]	3.30 ± 0.18[a]	3.10 ± 0.17[a]	2.15 ± 0.23[b]
Globulin (calc.)	g/dL	3.13 ± 0.17	2.62 ± 0.14	2.67 ± 0.13	3.05 ± 0.17
Total Bilirubin	mg/dL	0.20 ± 0.01	0.20 ± 0.01	0.19 ± 0.02	0.20 ± 0.01
Creatinine	mg/dL	0.50 ± 0.06	0.52 ± 0.05	0.44 ± 0.04	0.35 ± 0.06
Alkaline Phosphatase	IU/L	135.25 ± 40.61	62.67 ± 33.16	50.14 ± 30.70	91.75 ± 40.61
ALT	IU/L	510.25 ± 233.89	189.83 ± 190.97	762.14 ± 176.80	421.50 ± 233.89
Amylase	IU/L	55.00 ± 21.27	72.67 ± 17.36	62.71 ± 16.08	108.50 ± 21.27
AST	IU/L	132.25 ± 34.47	108.67 ± 28.14	159.71 ± 26.06	116.0 ± 34.47
Calcium	mg/dL	8.93 ± 0.19[ab]	9.27 ± 0.16[a]	8.9714 ± 0.15[a]	8.25 ± 0.20[b]
Cholesterol	mg/dL	355.25 ± 45.46	307.83 ± 37.12	257.43 ± 34.36	222.00 ± 45.46
CK	IU/L	590.50 ± 161.62	439.33 ± 131.96	582.29 ± 122.17	649.0 ± 161.62
Glucose	mg/dL	140.75 ± 23.95	99.50 ± 19.55	130.29 ± 18.10	139.25 ± 23.95
Magnesium	mg/dL	2.18 ± 0.10	2.30 ± 0.08	2.31 ± 0.08	2.30 ± 0.10
Phosphorus	mg/dL	4.55 ± 0.43	4.62 ± 0.35	3.93 ± 0.33	4.35 ± 0.43
Urea nitrogen	mg/dL	20.00 ± 1.68	22.00 ± 1.37	17.43 ± 1.27	16.25 ± 1.68
SDH	U/L	5.68 ± 2.80	6.52 ± 2.29	8.30 ± 2.12	7.83 ± 2.80
Osmolality (calc)	mos/kg	319.48 ± 1.82	319.05 ± 1.49	318.31 ± 1.38	322.03 ± 1.82
Serum Iron	µg/dL	233.00 ± 31.56	202.50 ± 25.77	216.71 ± 23.86	212.00 ± 31.56

[1] Data are presented as mean ± standard error (SE) of the mean.

[2] Means in the same row with different letter superscript are significantly different ($p \leq 0.05$).

Table 3-6. Effects of exposure to dietary PCB 126 on hematological parameters for seven-month-old mink.

Parameter	Unit	Dietary Concentration (µg PCB 126/kg feed) [1, 2] 0 (n = 8)	0.24 (n = 14)
White Blood Cell Count	x10.e3/µL	5.85 ± 0.90	6.62 ± 0.69
Red Blood Cell Count	x10.e6/µL	9.63 ± 0.18[a]	8.90 ± 0.14[b]
Hemoglobin	g/dL	18.01 ± 0.35[a]	16.84 ± 0.27[b]
Hematocrit	%	57.79 ± 1.15[a]	54.35 ± 0.88[b]
Mean Corpuscular Volume	fL	60.03 ± 0.42	61.04 ± 0.32
Platelets		603.29 ± 45.26	671.75 ± 34.57
Neutrophils	cells/µL	49.77 ± 5.51	46.01 ± 4.21
Lymphocytes	cells/µL	45.17 ± 5.57	49.35 ± 4.26
Monocytes	cells/µL	1.96 ± 0.33	1.56 ± 0.25
Eosinophils	cells/µL	2.61 ± 0.32	2.49 ± 0.24
Basophils	cells/µL	0.16 ± 0.02	0.12 ± 0.02

[1] Data are expressed as means ± standard error (SE) of the mean.
[2] Means in the same row with different letter superscript are significantly different ($p \leq 0.05$).
Male and female juvenile mink are grouped by treatment, because no significant differences were determined between the two sexes.

Table 3-7. Effects of dietary PCB 126 on serum chemistries in juvenile mink exposed *in utero* through seven months of age.

Parameter	Unit	Dietary Concentration (µg PCB 126/kg feed) [1, 2]	
		0 (n = 8)	0.24 (n = 14)
Sodium	mmol/L	152.86 ± 0.49	153.75 ± 0.38
Potassium	mmol/L	4.19 ± 0.10[a]	4.50 ± 0.08[b]
Chloride	mmol/L	112.00 ± 0.74	112.33 ± 0.56
TCO2	mmol/L	23.14 ± 1.21	24.67 ± 0.92
Anion Gap	mmol/L	21.90 ± 1.12	21.25 ± 0.86
Total Protein	g/dL	6.24 ± 0.14	6.16 ± 0.11
Albumin	g/dL	3.63 ± 0.12	3.58 ± 0.09
Globulin (calc)	g/dL	2.61 ± 0.14	2.56 ± 0.11
Total Bilirubin	mg/dL	0.20 ± 0.01	0.18 ± 0.01
Creatinine	mg/dL	0.54 ± 0.047	0.47 ± 0.04
Alkaline Phosphatase	IU/L	62.57 ± 3.96[a]	51.33 ± 3.02[b]
ALT	IU/L	150.86 ± 37.85	239.67 ± 44.84
Amylase	IU/L	67.29 ± 3.09	69.92 ± 2.36
AST	IU/L	107.71 ± 16.33	100.00 ± 12.47
Calcium	mg/dL	9.76 ± 0.15	9.83 ± 0.11
Cholesterol	mg/dL	335.43 ± 11.59[a]	258.67 ± 8.85[b]
CK	IU/L	561.57 ± 98.26	456.83 ± 75.04
Glucose	mg/dL	141.29 ± 5.88[a]	106.67 ± 4.49[b]
Magnesium	mg/dL	2.36 ± 0.04	2.38 ± 0.03
Phosphorus	mg/dL	4.64 ± 0.23	4.66 ± 0.18
Urea Nitrogen	mg/dL	19.57 ± 2.28	19.92 ± 1.74
SDH	U/L	8.94 ± 2.73	4.77 ± 2.09
Osmolality (calc.)	mos/kg	320.54 ± 1.11	320.54 ± 0.85
Serum Iron	µg/dL	197.29 ± 14.12	216.08 ± 10.79

[1] Data are presented as means ± standard error (SE) of the mean.

[2] Means in the same row with different letter superscript are significantly different ($p \leq 0.05$).

trial. This equates to 5.0 μg TEQ/kg feed since TCDD is used as the standard chemical to which all other TCDD-like PCB congeners are compared, and thus, has a toxic equivalency factor of 1.0 (Van den Berg et al. 1998). Aulerich et al. (1987) reported 50% mortality in mink exposed to PCB 169 for 135 days at a concentration of 50 μg/kg feed, whereas no deaths occurred at 10 μg PCB 169/kg feed in the same study. Since PCB 169 has a TEF value of 0.01, mortality occurred at a concentration of 5.0 μg TEQ/kg feed, compared to the current study in which mink were exposed to 2.4 μg TEQ/kg feed via the diet containing 24.0 μg PCB 126/kg feed.

Clinical Signs of Toxicity:

The clinical signs induced by PCB 126 were similar to signs previously reported for PCB toxicity in mink, such as "wasting syndrome" and disrupted molting patterns. Mink exposed to the two greater concentrations of PCB 126 showed signs of "wasting syndrome", a PCB-induced toxicity that has been well documented (Aulerich et al. 1987; Hochstein et al. 2001). Aphagia, or the inability to eat, appeared to be associated with the jaw lesion, described as squamous epithelial proliferation in the mandibles and maxillae (Chapter 4). This condition was followed by clinical detection of periodontoclasia first observed in mink in the highest dose group. This condition hindered feed consumption because the animals could not chew the food.

Animals in all PCB 126 dietary treatments expressed anorexia, melena, poor coat condition, increased shedding (inconsistent with natural patterns and timing of a seasonal molt), eye infections, and nasal discharge. The latter two conditions are suggestive of chronic low-grade infection, possibly resulting from immunosuppression

as suggested by the changes in the leukocyte differentials described below. The immune system is a target for PCB toxicity, and PCBs have been reported to cause immunosuppression (Bleavins et al. 1983; Safe 1990; Faustman and Omenn, 2001).

Nail Growth Abnormalities:

Dietary exposure to PCB 126 resulted in orthokeratotic hyperkeratosis of the toenails of mink. Clinical changes of the nails, including marked thickening, elongation, and deformity, were observed during daily monitoring. Growth appeared to vary between individuals, but was continuous. Only the front nails had abnormal growth. The nails of two mink were so long that they hindered the animals' ability to climb around the cage because the nails would get hooked on the wires. The growth characteristics (marked thickening, elongation, and deformity) of the toenails are comparable with results from other studies where mink were exposed to TCDD or PCB 126 (Hochstein et al. 1998, 2001; Render et al. 2000*a*, 2000*b*; Beckett et al. 2002).

Similar findings of abnormal nail growth in mink and European ferrets (*Mustela putorius furo*) exposed to PCBs have been reported in other studies. Mink exposed to 3,3',4,4',5,5'-hexachlorobiphenyl (PCB 169) at a dietary concentration of 50 µg/kg for 135 days, displayed pronounced nail growth, in addition to delayed molting patterns and altered guard hair growth and/or retention of underfur (Aulerich et al. 1987). Bleavins et al. (1982) reported that European ferrets (*Mustela putorius furo*) fed diets containing 20 mg/kg feed of the commercial PCB mixture Aroclor® 1242 for 266 days developed similar clinical signs of excessive nail growth. Histological examination of the feet and nails of ferrets indicated keratin debris adherent to the ventrum of the nail and in the

hair follicles, perivascular accumulation of mononuclear cells in the footpad dermis, nuclear indentations and prominent nucleoli in the stratum basale, and vacuolization of degenerating epidermal cells.

The toenail growth observed in the current study occurred within a shorter time span as compared to the aforementioned studies, suggesting that PCB 126 induced a more rapid and pronounced effect. Toenail growth reported for PCB 169 was observed at 19 weeks of exposure (Aulerich et al. 1987). Aroclor® 1242 induced nail growth in ferrets following 38 weeks of exposure (Bleavins et al. 1982). In the current study, excessive nail growth was first reported at five weeks of exposure to PCB 126, and growth continued throughout the study.

Body Weight Loss:

The decrease in body weight observed in the dams receiving 24.0 μg PCB 126/kg feed supports the occurrence of the characteristic PCB-induced "wasting syndrome". PCB-induced "wasting syndrome" was associated with toxicity signs in mink, which often occurred within the first month of the trial. Body weight loss associated with aphagia tended to occur after approximately two months of feed consumption. Body weight (BW) loss related to the inability to eat also correlated to the animal's interest in food and activity level during daily monitoring, as well as clinical signs of dental misalignment or gingival swelling or bleeding. Females with periodontoclasia were offered soft feed to overcome chewing difficulties. However, although there was not a statistically significant decrease in mean BW in PCB 126 exposed females compared to controls, dams in the 24.0 μg PCB 126/kg feed group lost

an average of 36% of their BW during the study period (initial BW to terminal BW), as compared to the mean BW loss in the control (8.7%), 0.24 μg PCB 126 (9.6%), and 2.4 μg PCB 126/kg feed (5.9%) groups over the same time period. The current study compares well with the study by Hochstein et al. (2001) that reported a mean BW loss of 26% in mink exposed to 5.0 μg TCDD/kg feed (Hochstein et al. 1998). Aulerich et al. (1987) also reported a significant decrease in BW in mink exposed to PCB 169, as a result of PCB-induced "wasting syndrome".

Organ Weights:

Adult females in the 24.0 μg PCB 126/kg feed group expressed a significant reduction in mean absolute heart weight compared to the control group (Table 3-2). Similarly, Hochstein et al. (1998) also reported a significant decrease in mean heart weight in animals fed 1.0 μg TCDD/kg feed compared to the control mink.

The liver comprises 4.9 to 5.1% of the total body weight of the adult mink (Aulerich et al. 1999). In addition to the significant increase in mean absolute liver weight observed in this study in the dams receiving 2.4 μg PCB 126/kg feed, there was a dose-related increase in liver weight expressed as percent of the total body weight (3.2, 3.6, 4.8, and 5.4% for the control, 0.24, 2.4, and 24.0 μg PCB 126/kg feed groups, respectively) (Table 3-2). Increased liver weight is a relatively common toxic response due to induction of hepatic cytochrome P-450 enzymes and possibly changes in fat metabolism in the liver of mink exposed to PCBs and TCDD (Aulerich et al. 1985; 1987; Bergman et al. 1992; Heaton et al. 1995; Hochstein et al. 2001). PCB- and TCDD-induced hepatocellular injury have been previously reported in mink (Platonow

and Karstad, 1973; Aulerich and Ringer, 1977; Bergman et al. 1992; Edqvist et al. 1992; Heaton et al. 1995), guinea pigs (reviewed in Safe 1990), and rats (Chu et al. 1994). Liver injury caused by toxic compounds is often due to the disruption of the metabolic capabilities of the hepatocytes that can lead to the following diseases, i.e., fatty liver, direct cytotoxic damage, cholestatic damage, cirrhosis, vascular lesions and hepatic neoplasms (Timbrell 1991).

Kidney Uroliths:

Kidney uroliths were observed during necropsy in one dam from each of the following groups: control, 2.4 and 24.0 μg PCB 126/kg feed groups. Uroliths are commonly referred to as (kidney) stones or calculi in the urinary tract, bladder and/or kidneys, and are associated with urolithiasis, a condition that is relatively common in ranch mink (Zellen 1996). As reported in the literature, stones are most frequently composed of magnesium ammonium phosphate (struvite) and occasionally calculi of calcium oxalate have been reported (Zellen 1996). Thus, it appears that PCB 126 does not alter the composition of kidney uroliths, and PCB 126 did not increase the incidence of acute urolithiasis. Mink frequently suffer from urinary tract infections, and may die without showing any signs of this condition, or they may have difficulty urinating (Aiello 1998).

Calculi recovered from PCB 126-exposed mink were analyzed for mineral content by the Minnesota Urolith Center (University of Minnesota, College of Veterinary Medicine, Department of Small Animal Clinical Sciences, St. Paul, MN). All specimens consisted of 90 to 100% magnesium ammonium phosphate hexahydrate

as the core of the urolith, and 0 to 10% of the shell consisted of calcium phosphate carbonate.

Hematology:

Exposure of mink to PCB 126 resulted in significant changes in hematological parameters in this study (Tables 3-4 and 3-6). In addition to our study, decreases in RBC counts, HGB, and HCT in mink have been reported in studies as a result of TCDD-exposure (Hochstein et al. 2001), as well as exposure to contaminated fish (Heaton et al. 1995) and rats (Kociba et al. 1976; Chu et al. 1994). An absolute decrease in RBC count and HGB and HCT concentration are indicative of anemia, where HCT is the most accurate measure (Duncan et al. 1994). Therefore, this would suggest that the dams and juvenile mink in the current study suffered from PCB 126-induced anemia. Mild to moderate anemia has been reported as a toxic response of PCB exposure in several species (Bergman et al. 1992).

Red blood cell indices may be used to help classify the type of anemia, which would suggest primary or secondary PCB-induced toxicity. Based on the RBC indices, the anemia observed in the current study may be suggestive of regenerative anemia based on the bone marrow response of the production and thus the presence of reticulocytes in circulation. Macrocytosis (a significant increase in MCV), hypochromia (a significant decrease in MCHC, but not MCH), and an increase in red cell distribution width (RDW) were observed in the current study, which are suggestive of this regenerative type of anemia (Tips 1993; Duncan et al. 1994). Macrocytic anemia is generally only associated with severe, chronic, progressive liver disease, and not

typically seen in acute (even when severe) liver disease (Ravel 1995). Mean corpuscular volume was significantly increased by PCB 126 exposure in the dams. In the juvenile mink, there was a non-significant ($p = 0.07$) increase in MCV in the PCB 126-treated group when compared to the control group. In addition, serum iron concentrations of the adult females in all of the PCB 126-treated groups were decreased as compared to the control group, but not significantly. This suggests that PCB 126 could cause hemolysis, but the mechanism is not understood. However, hemolytic states have not previously been reported as a toxic response following PCB exposure in mink or other species (Bergman et al. 1992; Edqvist et al. 1992).

Increases in total WBC counts were observed in a dose-related manner in adult (significant) (Table 3-4) and juvenile (non-significant) (Table 3-6) mink exposed to PCB 126 in the present study. This observation compares favorably with previous findings suggesting that leukocytosis is a common observation following PCB treatment in different species including mink (Gillette et al. 1987; Bergman et al. 1992). Neutrophilia was observed in the dams exposed to PCB 126 in this study. A change in the presence of neutrophils is an indication that the immune system may be having difficulty in overcoming a latent infection (Tips 1993).

A dose-related increase in platelets was observed in both the dams and the juvenile mink in this study, however, the increase was not statistically significant (Tables 3-4 and 3-6). Contrary to this finding of increased thrombocytes in the current study, Osweiler (1996) suggested that a toxicity-induced hematopoietic depression would cause thrombocytopenia. As stated above, rats exposed to PCB 126 also exhibited decreased thrombopoiesis, which is indicative of abnormal activity of the

bone marrow supported by histological findings (Chu et al. 1994). This suggests that there may be differences between species and their responses to PCB 126 exposure. Platelet number also can be affected be splenic activity, and biologically, there is a greater risk of hypercoagulation in animals with thrombocytosis (Duncan et al. 1994).

Serum Chemistries:

Several significant changes in serum chemistries were observed in the current study in both adult and juvenile mink exposed to PCB 126. Enzyme activity is an important measure of function and potential cellular injury, especially when it involves the hepatic system.

Serum ALT activity in juvenile mink was increased in the PCB 126-exposed group as compared to the control group (non-significant) (Table 3-7). In a study where mink were exposed to various commercial PCBs and PCB fractions, Edqvist et al. (1992) reported elevated ALT activity only in mink exposed to the commercial PCB compared to the control group. Hochstein et al. (2001) reported that the group of mink fed a diet containing 5.0 μg TCDD/kg feed had significantly increased ALT activity. Contrary to the ALT increases observed in mink, a study exposing guinea pigs to TCDD found decreased ALT activity (reviewed in Safe 1990), which might be suggestive of a decreasing number of hepatocytes due to the liver undergoing fibrosis, or because there are fewer hepatocytes in general (Duncan et al. 1994). The enzyme ALT is a more sensitive indicator of chronic progressive hepatic disease, and concentrations remain elevated in serum longer compared to AST (Tips 1993; Duncan et al. 1994). Changes in ALT activity are indicative of liver function, and more

specifically may reflect hepatocellular injury as a result of chronic hepatic disease or an altered state of hepatocellular integrity as well as a decrease in hepatocyte number (Tips 1993; Duncan et al. 1994; Ravel 1995).

A significant decrease in alkaline phosphatase (ALP) was observed in the juvenile mink receiving the PCB 126 diet. Similar findings were also reported for mink fed commercial PCBs (Edqvist et al. 1992). Hochstein et al. (2001) reported a numerical decrease in ALP activity in mink fed TCDD. However, it was reported that alkaline phosphatase activity in rats significantly increased following exposure to PCB 126 (Chu et al. 1994). Alkaline phosphatase is an enzyme that is primarily found in liver, bone, placenta, and intestinal mucosa. Thus, when these tissues are damaged, increased amounts of ALP are released into the bloodstream. When this increase of ALP is due to liver disease, it is usually associated with cholestasis, or obstruction of bile flow (Duncan et al. 1994).

However, in agreement with Edqvist et al. (1992), cholestasis is probably not extensive in the current study based on the decreased ALP activity observed in mink. As aforementioned, some degree of cholestasis is invariably associated with hepatocellular damage or injury. For example, based on swelling that can accompany hepatocellular injury, compression of the bile canaliculi can lead to some degree of cholestasis (Tips 1993; Duncan et al. 1994). Decreases in ALP activity, as observed in the present study and in the study by Edqvist et al. (1992), can also be suggestive of low adrenal function, which would indicate impaired phosphate liberation during utilization of ATP for energy (Tips 1993).

Serum cholesterol was significantly reduced in juvenile mink exposed to PCB 126. In addition, cholesterol was substantially decreased (non-significantly) in a dose-related manner in adult females exposed to dietary PCB 126. Edqvist et al. (1992) reported that the non-*ortho* PCB fractions were most active in reducing the concentration of cholesterol in mink, which corresponds with the coplanar structure of PCB 126 used in the current study that significantly reduced serum cholesterol observed in both juvenile and adult mink. Hochstein et al. (2001) also reported significant reductions in serum cholesterol in mink that had been treated with TCDD. Contrary to the decreases in cholesterol observed in mink, increases in serum cholesterol have been reported in rats, presumably due to enhanced cholesterol production (Chu et al. 1994). In mink, a reduced synthesis of cholesterol may be due to decreased functional mass of the liver leading to hypocholesterolemia, and this decrease could be viewed as a health concern, particularly since it can indicate poor digestion of fats. The effects of PCBs on cholesterol may be indicative of altered fat metabolism as a result of PCB-induced hepatic dysfunction.

Albumin, which comprises 35-50% of the total serum protein (Duncan et al. 1994), was significantly reduced in the dams receiving the diet with 24.0 μg PCB 126/kg feed in the present study. A significant decrease in albumin was also reported in mink exposed to TCDD (Hochstein et al. 2001). Chronic cachectic or "wasting diseases" may contribute to hypoalbuminemia, although it is unclear whether the mechanism leading to the decrease in albumin is due to impaired synthesis or other factors (Ravel 1995).

The decreases in BUN observed in mink were not significant ($p = 0.07$) for dams, and no changes in BUN levels were observed in juvenile mink fed PCB 126 in this study. However, BUN is an important indicator of hepatic function and injury, and the reduced BUN concentrations in the adult mink may indicate hepatic insufficiency, and may be viewed as a precursor to hepatic injury. Contrary to the current study, Edqvist et al. (1992) reported azotemia in mink treated with PCBs. The cause(s) for this difference is unknown but may correlate with previous pathological observations in mink is postrenal (obstructional) azotemia caused by ureteral or urethral obstruction by uroliths.

Significant changes were observed in various electrolytes (Na, K, Cl, and TCO_2) and AG in the dams and the juvenile mink exposed to PCB 126 in the present study. Hyperkalemia was significant in the juvenile mink in the PCB 126 group, and increased, but not significantly, in the dams receiving 24.0 µg PCB 126/kg feed. Potassium concentration can increase in the blood as a result of RBC hemolysis, as in the case of severe hemolytic anemia (Ravel 1995). The observation of anemia in mink exposed to PCB 126 in this trial is supported by the hematological data previously presented. Clinical signs for hyperkalemia may include muscle weakness, nausea, anorexia, and mental changes (tend toward drowsiness and lethargy) (Ravel 1995).

Significant hypocalcemia was observed in dams consuming the PCB 126 diet. Mink exposed to Aroclor 1254 were also reported as having decreased concentrations of serum calcium (Edqvist et al. 1992). Hypocalcemia is often associated with hypoalbuminemia and alkalosis in most species, as well as hypoproteinemia (Duncan et al. 1994).

Increased chloride concentration was observed in the females on the highest PCB 126 diet as compared to the other PCB 126 dose groups. Generally, hyperchloridemia follows the direction of changes in sodium ion gradient (Duncan et al. 1994; Ravel 1995). Biologically, maintaining the chloride concentration is essential, as chloride is an important component of secretions (saliva and gastric secretions) (Duncan et al. 1994). In addition, the significant decrease in serum TCO_2 that was observed in dams on the PCB 126 diet suggesting metabolic acidosis correlates with the observed increase in chloride ion concentration in the dams.

Anion gap significantly decreased in dams fed the PCB 126 diet. Anion gap changes were not significantly changed in the juvenile mink in this study, as was also reported in mink exposed to TCDD (Hochstein et al. 2001).

Serum glucose concentrations were significantly reduced in the juvenile mink fed the PCB 126 diet, whereas the dams on the PCB 126 diets had no changes in glucose concentrations as compared to controls. In other studies, glucose concentrations were decreased in mink exposed to PCBs (Edqvist et al. 1992), and TCDD (Hochstein et al. 2001), and in rats exposed to PCB 126 (Chu et al. 1994). Glucose metabolism can become disrupted through various mechanisms: the liver fails to breakdown glycogen, the absorption of glucose in the intestines is impaired, insulin receptors are reduced, or insulin production is reduced as a result of a decline in pancreatic beta cells (Tips 1993).

Other serum chemistry changes included decreased creatinine levels in both dams and juvenile mink on the PCB 126 diets, which could be suggestive of muscle wasting (Ravel 1995). Bilirubin was unchanged in females, and mildly lowered in juvenile mink on the PCB 126 diet.

Due to changes observed in various blood clinical parameters that can be associated with pancreatic function, it would prove highly valuable to histology evaluated pancreatic tissue in future studies. Changes observed in this study included mild hyperamylasemia (females, $p = 0.09$), neutrophilia, increased ALT, hyperglycemia, and mild hypocalcemia.

CONCLUSIONS

In conclusion, this study reports that the dietary consumption of PCB 126 produced a multitude of toxic effects in adult female and juvenile mink including clinical, hematological, and biochemical changes. Although the majority of the effects occurred at a dietary concentration of 24.0 μg PCB 126/kg feed, effects were seen in all dose groups, including the juvenile mink receiving the diet containing 0.24 μg PCB 126/kg feed.

A review of biological parameters correlated several of the observed hematological and serum chemistry parameters from this study and others with the pathologic and clinical changes that have been reported for PCB-induced toxicity. In doing so, data have also shown distinct species differences in the biochemical responses to PCB-induced toxicity. The results from this study strongly suggest that these changes in hematology and serum chemistry are biologically relevant effects of PCB 126, and are consistently observed in mink exposed to PCBs and TCDD. This demonstrates the highly toxic potential of PCB 126 and supports the sensitivity of the mink to PCBs.

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CHAPTER 4

PCB 126-Induced Oral Squamous Cell Carcinoma of the Mandible and Maxilla in Mink (*Mustela vison*)

PCB 126-Induced Oral Squamous Cell Carcinoma of the Mandible and Maxilla in Mink (*Mustela vison*)

ABSTRACT

Mandibular and maxillary squamous epithelial proliferation was induced experimentally in mink (*Mustela vison*) by 3,3'4,4',5-pentachlorobiphenyl (PCB 126), a ubiquitous contaminant, which is structurally similar to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). The histopathological appearance of this lesion suggests squamous cell carcinoma (SCC). The characteristics of the lesion observed in mink from the current study were consistent with some of the criteria for malignant neoplasia, including invasiveness of atypical cell type, tissue destruction, increased proliferative activity, and diffuse infiltration. However, additional criteria needed to be satisfied in order to classify the lesion as malignant neoplasia. The current study was designed to determine if a PCB 126-induced lesion in the gingival tissue of mink was cancerous by satisfying two additional criteria of carcinogenicity. These included (1) the progression of the lesion after removal of the stimulus (PCB 126); and (2) the ability of the cells comprising the lesion to grow and form a tumor after being transplanted into athymic mice. The lesion was histologically present in mink euthanized after only two weeks of consuming a diet containing 24.0 μg PCB 126/kg feed. The lesion was clinically detectable in mink exposed to the PCB 126 diet for three, four, and five weeks following 12 weeks post-exposure. In addition, the lesion progressively worsened during the six or eight months post-exposure period (classified the non-PCB period) in animals fed diets containing PCB 126 for one to six weeks, thus meeting the first

criteria of progression after withdrawal of the PCB 126 stimulus. In the nude, athymic mouse trial, nodular growths were observed in two of the 18 mice that were implanted with gingival tissue from the oral lesion of mink exposed to PCB 126. Although the nodules in the mice were classified as non-malignant, the origin was non-host (mink) stratified squamous epithelium, demonstrating consistency with neoplastic criteria. Both objectives of this study were met, therefore meeting additional criteria for carcinogenicity. This is the first report of PCB 126-induced oral SCC in mink.

INTRODUCTION

Chemically-induced carcinogenesis, caused by exposure to chemicals such as polychlorinated biphenlys (PCBs) and 2,3,7,8-tetrachorodibenzo-*p*-dioxin (TCDD), is a multi-stage process that can lead to subsequent neoplasia (Safe 1989; Whysner and Williams, 1996; Gregus and Klaassen, 2001; Klaunig and Kamendulis, 2001; Cockerell and Cooper, 2002). Chemical carcinogens may affect multiple cellular and molecular mechanisms that alter normal function of cells at various levels, leading to neoplastic changes (Pelkonen and Nebert, 1982; Silberhorn et al. 1990; Klaunig and Kamendulis, 2001). Carcinogens can be classified into two broad categories, genotoxic and epigenetic chemicals, based on their chemical attributes in the process of carcinogenesis.

These terms "genotoxic" and "epigenetic" chemicals were identified by Williams and Weisberger (1983) to recognize the apparent differences of these

chemicals that induced neoplasia and to describe their possible mode of action (Klaunig and Kamendulis, 2001). Genotoxic chemicals generally cause direct damage to genomic DNA, and are activated in the target cell. Genotoxic compounds are capable of producing a preneoplastic change following a single administration, which is generally at or close to the site of chemical exposure (Klaunig and Kamendulis, 2001). Much less is known about the mechanism(s) of epigenetic carcinogens, although they do not appear to cause damage to DNA directly (Klaunig and Kamendulis, 2001). However, these non-genotoxic agents appear to modulate cell proliferation and apoptosis, and may act by producing an early proliferative response (mitotic activity) that is reversible following removal of the test chemical stimulation (Klaunig and Kamendulis, 2001; Boatman et al. 2004). To elicit neoplasia, exposure to epigenetic chemicals must be chronic and there is a threshold dose (Klaunig and Kamendulis, 2001; Boatman et al. 2004).

Neoplasia can be categorized as benign or malignant neoplasia. However, a neoplasm can defy categorization (Cotran et al. 1999). Benign and malignant neoplasms are differentiated by morphological and behavioral characteristics. Benign neoplasms are non-cancerous and generally localized, with limited potential for growth and slow growing in nature. These tumors are composed of cells that are well differentiated, do not invade surrounding tissue, and do not metastasize to distant sites. Benign tumors adjacent to bone may cause cortical erosion in a response to local pressure, without actually invading the bone (Rosenberg 1999; Thompson and Pool, 2002). Malignant neoplasms tend to grow rapidly, may contain anaplastic cells, and can metastasize to other organ sites depending to their site of origin. Malignant neoplasms

characteristically invade surrounding tissue, and tend to be more aggressive and destructive, therefore they are often more osteolytic than benign neoplasms (Konde 1994; Myer 1994; Aiello 1998; Cotran et al. 1999; Thompson and Pool, 2002).

Malignant soft tissue neoplasms of the oral cavity often involve adjacent bone (Dennis 1991; McGlennon 1991; Myer 1994; Cotran et al. 1999; Cockerell and Cooper, 2002; Thompson and Pool, 2002). Osteolysis may be focal or diffuse, and may involve the mandible, maxilla, premaxilla, or hard palate (Dennis 1991). In the oral cavity, the lamina dura is a sheet of compact alveolar bone that lies adjacent to the periodontal membrane, which is the lining of the tooth socket. Tissues adjacent to alveolar bone, such as the gingiva and the periodontal ligament, can be invaded by epithelial cells, which can lead to osteolysis (Birek et al. 1983). Invasiveness is a hallmark of malignant neoplasia, along with metastases, and it is the most reliable feature that differentiates malignant from benign tumors (Cotran et al. 1999).

Carcinogenicity of PCBs has been demonstrated in numerous rodent studies reviewed by Safe (1989), Erickson (1997), Smith (1997), and Cogliano (1998). However, animal-feeding studies that investigated the carcinogenic potential of PCBs focused primarily on commercial PCB mixtures (Erickson 1997; Cogliano 1998). Commercial mixtures with higher chlorine content, such as Aroclor 1260 that contains 60% chlorine, are suggested to have a higher neoplastic potential (Cogliano 1998). Therefore, it is not known if a specific PCB congener in the commercial mixtures is responsible for these effects or if there are additive effects, decomposition products, contaminants, or metabolites that are involved in the toxic response (Erickson 1997). Very few studies have been conducted with specific PCB congeners (Cogliano 1998).

In fact, only one PBC congener, 2,2',3,3',6,6'-hexachlorobiphenyl, had been studied and the results indicated no significant neoplastic effect (Cogliano 1998).

Studies that investigated the carcinogenic potential of PCBs and TCDD have also examined their activity as initiators and/or as tumor promoters (Safe 1989). 2,3,7,8-Tetrachorodibenzo-*p*-dioxin has carcinogenic potential by acting as a promoter of neoplastic activity, and this may have resulted in an increased incidence of squamous cell carcinoma in rats and hamsters (Kociba et al. 1978; Whysner and Williams, 1996). Studies have shown that PCB 126 has tumor-promoting activity in liver and lung tissues (Safe 1989; Silberhorn et al. 1990; Cogliano 1998). Thus, PCB 126 could be a tumor-promoting agent in other tissues, including the gingiva (only liver, brain, mammary gland, and male thyroid gland were investigated in the cited group of studies) (Cogliano 1998).

Our laboratory has reported a TCDD- and PCB 126-induced lesion in juvenile mink (*Mustela vison*) identified as mandibular and maxillary squamous epithelial proliferation (Render et al. 2000*a*, 2000*b*, 2001). The lesion is characterized by proliferation of squamous cells that form nests and/or cords that infiltrate into the periodontal ligament and alveolar bone causing osteolysis of the mandible and maxilla. The histological appearance of this jaw lesion is suggestive of squamous cell carcinoma (SCC). Standard dark, female mink (*Mustela vison*) were used to determine if the jaw lesion observed in mink was in fact oral carcinogenicity induced by dietary exposure to PCB 126.

Specific criteria of malignant neoplasia, including higher proliferative activity, tissue destruction, and invasiveness of atypical cell type and diffuse infiltration (Cotran

et al. 1999), are conditions that are consistent with characteristics of the jaw lesion observed in mink exposed to PCB 126. Thus, this study was designed to determine if the jaw lesion in mink would meet additional criteria of malignant neoplasia, including progression or regression after withdrawal of the stimulus (the PCB 126-contaminated diet), and the formation of a tumor from a tissue transplant or injection of cells into a suitable host animal (nude, athymic mice). No prior carcinogenic studies in mink using PCB 126 studies have been conducted. Therefore, it was hypothesized that PCB 126 induces squamous cell carcinoma in the maxilla and mandible of mink. The first objective was to determine if the histologically detectable lesion in the maxilla and mandible of mink would progress or regress in exposed animals after withdrawal of the diet containing 24.0 μg PCB 126/kg feed. Female mink were provided one, two, three, four, or five weeks of exposure to the PCB 126 diet followed by a 6-month withdrawal period. A separate experiment group was exposed to the PCB 126 diet for six weeks followed by an 8-month withdrawal period. The second objective was to determine if the squamous epithelial hyperplasia previously reported in mink exposed to 24.0 μg PCB 126/kg feed was in fact SCC, by extracting gingival tissue from the oral lesion and transplanting or injecting the pathologic cells into athymic mice, thus producing a tumor growth.

METHODS

Standard dark, female mink were used as an animal model to assess the oral carcinogenicity of PCB 126. All mink were housed individually in wire mesh cages (61 cm L x 76 cm W x 46 cm H) with an attached wooden nest box (30 cm L x 22.5 cm W x 25 cm H) at the Michigan State University (MSU) Experimental Fur Farm containment facility. Lighting in the room was maintained to simulate the natural light/dark cycle, of the Eastern Standard Time zone. Mink were acclimated for one week after being moved into the containment facility. Females were randomly assigned to a designated treatment group, except that litter-mates were not placed within the same treatment group. Mink were monitored daily for the duration of the study for any signs of toxicity (lethargy, inappetence, body weight loss, behavior alterations, and teeth displacement) and weighed weekly. If the jaw lesion progressed to a stage where a mink was unable to eat, or the mink was clinically unhealthy, the animal was euthanized and the jaws and major organs (adrenal glands, brain, heart, liver, kidneys, reproductive tract, spleen, and thyroid glands) were collected and processed for subsequent histopathological examination.

Diet - PCB 126-Supplemented Feed:

Complete methods for diet preparation are previously described in Chapter 2. Briefly, a standard ranch mink diet (Table 2-1) was supplemented with 24.0 μg PCB 126/kg feed and was designated the treatment diet. The standard ranch diet without supplemental PCB 126 was used as the control diet. The diets were aliquoted into two-

liter, plastic containers that contained enough feed for one to two days per diet. Feed was stored frozen and thawed in a cooler when required. Fresh thawed food was feed each day at approximately 140 g per female per day. Water was available *ad libitum.*

Lesion Classification:

Observations were used to clinically classify the jaw lesion in mink. Initially, swelling of the muzzle was observed, and gingival tissue appeared red and inflamed. Mild changes in dentition alignment included increased interdental space between the incisors and incisor unevenness within the mandibles and/or maxillae. Periodontoclasia, or the loosening of permanent teeth due to the breakdown and absorption of the supporting bone, and tooth displacement varied from subtle to severe with complete tooth loss. Occasionally, the incisors appeared to be very thin, suggesting a decrease in the enamel layer. Upper canine teeth appeared extended probably due to the receding of the gums and the loss of support from around the root of the tooth, including the periodontal ligament and the alveolar bone, allowing the tooth to loosen and extend out of the socket. With severe progression of the lesion, mink had bleeding and swollen gums, and nodular growths that were evident along the dental arches.

Histologically, jaw lesions were rated from mild to severe based on the following criteria: mild was characterized by one or a few small, focal cyst(s) that are considered to be the initial stage of the lesion. A lesion that was classified as moderate in severity consisted of either several foci of cysts and islands of squamous epithelial cells, or fewer large cysts that invaded the periodontal ligament and disrupted the teeth. A severe lesion consisted of several cysts of increasing size and invasiveness and has

progressed throughout the mandible and/or maxilla, destroying all surrounding tissues including the underlying alveolar bone that supports the teeth.

Two experiments were conducted to determine if the histologically detectable jaw lesion progressed or regressed after withdrawal of mink from the diet containing PCB 126. The two experimental procedures differed by number and age of animals, as well as exposure regimen. Both trials took place at the MSU Experimental Fur Farm in the containment facility, and mink were fed the same PCB 126 dietary concentration, 24.0 μg PCB 126/kg feed.

In *Experiment 1,* four young (8-months of age) female mink were randomly assigned to each of the five exposure groups, with no group having more than one animal from the same litter. Mink were fed a diet containing 24.0 μg PCB 126/kg feed for one, two, three, four, or five weeks. At the end of each exposure period, two mink from each group were placed on clean ranch feed for six months to assess lesion development, and the other two mink from each group were euthanized to examine the jaws histologically for evidence of the jaw lesion.

In *Experiment 2*, 20 female mink comprised of juvenile (6-months of age) and adult (18-months of age) were assigned to either the control group (five young and five adult mink) or the treatment group, which were fed a diet containing 24.0 μg PCB 126/kg feed (five young and five adult mink). The mink that were randomly assigned to the treatment group receiving the PCB 126/kg feed were fed the diet for a 6-week period. Following the period of exposure to the PCB 126-supplemented diet, two control (one young, one adult) and two treatment (one young, one adult) mink were euthanized to determine the histopathological prevalence of the lesion. The remaining

eight PCB 126-exposed mink were taken off contaminated feed and maintained on a clean ranch diet for an additional eight months (38 weeks) to determine progression or regression of the lesion.

Necropsies:

At the end of six (Experiment 1) or eight (Experiment 2) months, mink were euthanized using CO_2, and a gross examination was performed. Organs (adrenal glands, brain, heart, kidneys, liver, reproductive tract, spleen, and thyroid glands) were examined, collected and weighed, and sections were fixed in 10% buffered formalin (pH 7.4). Approximately 10 g of the liver were frozen (–74°C) immediately after extraction for subsequent PCB 126 analysis. Organ tissues were trimmed, embedded in paraffin, sectioned to 5 μm, mounted on microscopic slides, and stained with hematoxylin and eosin (H & E) for subsequent histological assessment.

At necropsy, the maxilla and mandible were collected from each female and fixed in 10% buffered formalin (pH 7.4). The jaws were decalcified in Surgipath® Decalcifier II (hydrochloric acid) (Surgipath Medical Industries, Inc., Richmond, IL) and trimmed. The decalcified tissues were then processed for histological assessment, which included paraffin embedding, sectioning, and staining with H & E for light microscopic examination. Photomicrographs of jaw histopathology are presented in color in the Results Section of this chapter and other sections of this dissertation.

PCB 126 Analysis of Mink Livers:

Following necropsies of mink, one or two liver samples were pooled from each exposure period and placed in I-Chem® jars (New Castle, DE). Approximately 8 g of liver (wet weight) were homogenized with anhydrous sodium sulfate and Soxhlet extracted with dichloromethane and hexane (3:1, 400 mL) for 16 hr. PCB-30 (17.5 ng) was added as an internal standard prior to extraction, for the first five samples and a blank, and PCB 30 and PCB 204 (17.5 ng each) were added as internal standards for the second set of six samples. Extracts were then concentrated to 6 ml and 0.5 mL was used for the analysis of lipid content by gravimetry. The remaining extract was then treated with concentrated sulfuric acid for the removal of lipids. Extracts were then passed through a multi-layer silica gel column (2 g 100-200 mesh, 2 g of 40% acidic silica, followed by another 2 g of 100-200 mesh silica gel; Davisil (Sigma, St. Louis, MO). The congener PCB 126 was eluted with 5% dichloromethane in hexane (100 mL), and the extract was concentrated to 1 mL, treated with concentrated sulfuric acid and injected into an Agilent 6890N gas chromatograph with 7683 autosampler, equipped with a ^{63}Ni electron capture detector (Boston, MA). A fused silica capillary column (30 m x 0.25 mm i.d.) coated with ZB-5 (5% phenyl polysiloxane) at 0.25 μm film thickness was used for the separation. The column oven was programmed from an initial temperature of 50°C (1 min hold) to 180°C at a rate of 20°C/min, held for 3 min, and then ramped at a rate of 2.5°C/min to 280°C with a final hold time of 8 min. The injector and detector temperatures were maintained at 250°C and 300°C, respectively. Helium and nitrogen were the carriers and the make-up gases, respectively.

3,3',4,4',5-Pentachlorobiphenyl was quantified using an external, standard prepared at a calibration range of 1 ng/mL to 100 ng/mL. Recoveries of PCB 30 and PCB 204 through the analytical method ranged from 60 to 88% (means: 68 and 70%, respectively). Concentrations of PCB 126 were corrected for the recoveries of PCB 30 and PCB 204. The detection limit for PCB 126 was 50 pg/g, wet weight (K. Kannan, *personal communication*).

In *Experiment 3*, four standard dark, juvenile, female mink (6-months of age) were fed a diet supplemented with 24.0 μg PCB 126/kg feed to induce neoplasia of squamous epithelium in the mandible and maxilla, while two mink that served as controls were fed a standard ranch mink diet. Procedures for Experiment 3 (housing, animal assignment, acclimation and mixing of diets) followed the methods as stated above. Fresh thawed feed was provided every day at approximately 140 g per female per day. Water was provided *ad libitum*. Mink were observed daily. Mink remained on treatment until clinical determination of the oral lesion.

Once the lesion was clinically classified as severe, mink were euthanized using CO_2. The gingival tissue was harvested using sterile razor blades. Following gingival harvest, the mandible and maxilla were collected and processed for histological examination as previously described. Extraction of oral tissue from the two control mink and implantation of tissue followed the same protocols as for the exposed mink.

Extracted tissue was placed into Modified Eagle's Medium (MEM) solution (Eagle's minimum essential [Eagle's ASP] medium supplemented with L-aspartic acid [0.2 mM], L-serine [0.2 mM], and pyruvate [1.0 mM]). Gentamicin (GIBCO-Invitrogen Corp., Carlsbad, CA) (0.05 mg/mL) was then added to the MEM solution. After

complete extraction of the mink lesion, all tissue pieces were placed in three independent washes of MEM. Tissue was then placed in a fresh vial of MEM solution for approximately 20 minutes. All surgical instruments were sterilized at 250°C for procedures using an Ionotec® Bead Sterilizer (Runcorn, Cheshire, England).

Nude, athymic mice (both sexes) were approximately 5-weeks of age at the time of the procedure. Three mice per affected mink were used for the transfer of gingival cells. Two methods were used to deposit the gingival cells from the mink lesion into the right and left flank of each mouse. The first procedure involved transplanting sections of gingival tissue, which were approximately two to three mm^2. The second procedure involved injection of a single cell suspension derived from the oral lesion cells. Both the transplant and the injection procedures were carried out under a laminar flow hood to maintain a sterile environment. Mice were first anesthetized using 0.5 to 1.0% isoflurane with oxygen at 0.3 liter/min (Bickford Vapomatic Anesthetic Vaporizer for Isofluorane Company, Wales Center, NY). The mouse's back was prepared for the surgical procedure by a 3-step cleaning process that involved swabbing with a sterile ethanol swab, swabbing with a sterile cotton swab with Novassan, and then wiping it with a sterile cotton bud with sterile water.

The toe reflex test that checks for muscle relaxation was conducted to ensure that the animal was maintained under deep anesthesia. An incision approximately 1.0 cm in length over the lower spinal column was made using shears and holding with forceps through the skin layer only. The skin was dissociated from the muscle layer to create a pocket, one on each flank. The lesion tissue was placed into the skin pockets

using sterile forceps. The incision was then stapled using a 9 mm autoclip, and swabbed with a sterile alcohol pad.

Single Cell Suspension Preparation:

A *Medimachine*™ (Becton Dickinson, Franklin Lakes, NJ) was used to disaggregate tissue into individual cells. Briefly, the single cell suspension was prepared by inserted four or five pieces of gingival tissue, approximately 2.5 mm^2 in size, into the *Medicons*™ (Becton Dickinson, Franklin Lakes, NJ) disposable capsule with 1 to 1.5 mL of buffering solution (PBS) with antibiotics. The *Medicons*™ was then inserted into the *Medimachine*™ housing and run for one minute. In standby mode, tissue was checked, and the *Medimachine*™ run for an additional 1 min. The *Medicons*™ was then removed from the machine. A sterile *Filcons*™ was applied to a 3.0 mL syringe in the housing of the *Medimachine*™, withdrawing the cellular suspension and placing it in a test tube. The cell suspension was centrifuged for five minutes at 800 rpm, forming a pellet of cells. The pellet was resuspended in 500 µL in solution containing 10 µg of metronidazole. The resuspended pellet was washed three times in metronidazole at 20 mg/mL. Following the washes, 1 mL of the cellular suspension was drawn into a syringe, and using a 25-gauge needle 500 µL was injected into sponges that had been surgically inserted subcutaneously on each flank of the mouse, one week prior to the injection.

Following both the cell injection and the tissue transplant procedures, the recovering mouse was returned to a clean, autoclaved cage with irradiated food and autoclaved water. All mice were monitored daily to ensure the surgical sites were

healing properly and that each mouse was in good health by monitoring robustness, pinkness of nose and skin color, alertness and active, and having no signs of morbidity or illness. Also, each mouse was individually tagged for identification with an individual ear notch. The staples were removed seven days after surgery, and the mice were monitored twice a week for tumor formation. The skin was palpated to check for small nodules, and any growths were measured to monitor growth rate. If a growth was present, the mouse was euthanized when the tumor reached 1 cm^3. The mice were euthanized at six months following transplant or injection if no growths developed or became apparent during this period.

RESULTS

The PCB 126-induced oral lesion in mink was histologically identified by specific characteristics. Nests and/or cords of proliferating stratified squamous epithelial cells destroyed gingival tissue with enlarging cysts and would also infiltrate into the adjacent periodontal ligament as well as the underlying alveolar bone. Some cysts had centers of exfoliating epithelia with possible keratin.

The lesion rating system that was developed for these studies is defined in the methods section. Briefly, a minimal to mild lesion consisted of one or a few small, focal nests of squamous epithelial cells that characterized the initial phase of the lesion. Invasion of alveolar bone had been detected, but not caused clinical signs of periodontoclasia. As the lesion progressed to a moderate rating, the number and size of

these islands of squamous cells generally increased, forming large islands of squamous epithelial cells or cysts. At this stage, the islands generally were more diffuse, and often several foci were found in more than one quadrant of the jaw. Also at this stage, nests and/or cords of squamous epithelial cells had invaded the periodontal ligament (PDL) and alveolar bone, resulting in changes to dental support structures and osteolysis. Clinical signs were visible at this stage. A severe rating based on histology was determined by the aggressive invasion of the cysts causing destruction of gingival tissue, loss of the soft-tissue attachment to the tooth (PDL) and severe osteolysis of the underlying alveolar bone. Clinical signs of periodontoclasia were visible, including spreading of incisors and often missing dentition. Also, individual variation appears to play a role in the susceptibility and the presentation of the lesion. Some family groups (siblings), even though on different treatment groups exhibited signs more quickly and more severely that other mink.

Clinical signs were evaluated in addition to the histological lesions, and thus used to rate the lesion and health status of the PCB 126-exposed mink. Although histological changes occur prior to clinical changes, clinical signs were observed visually. Clinically, swelling of the muzzle was observed, and gingival tissue appeared red and inflamed. Mild changes in dentition alignment included increased interdental spreading of the incisors, as well as periodontoclasia to tooth loss. With severe progression of the lesion, mink had swollen and bleeding gums, and nodular growths that were evident along the dental arches.

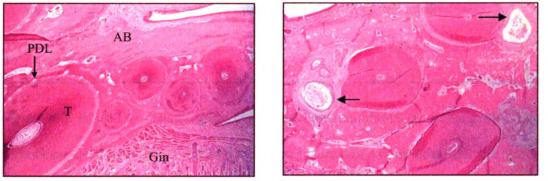
Nodular growths were observed above the oral palette in one mink exposed to PCB 126 for six weeks and then on clean feed for eight month, and in mink exposed

continuously for Experiment 3: the athymic mouse trial. Also noted was severe osteolysis of the nasal turbinates of mink in both of these treatment groups.

The histological results for exposure to PCB 126 in mink are organized by week, and include the weekly exposures (weeks one through six), and the post-exposure evaluation following the removal of the stimulus (PCB 126 diet) for six or eight months (Figures 4-1 through 4-6).

A) **PCB 126 Exposure for One Week** (Figure 4-1). 1) *Weekly exposure*: The two mink that were euthanized after one week of exposure to a PCB 126 diet exhibited no histological evidence of the lesion. 2) *Six months post-exposure*: following removal of the contaminated diet for six months, both mink were euthanized after receiving only one week exposure to the PCB 126 diet. One mink exhibited one focal cyst near the incisors of the mandible, thus, a minimal rating was assigned. The second mink exhibited moderate changes in all four quadrants of the jaw. In addition, multiple foci were cystic and diffuse in the gingival tissue.

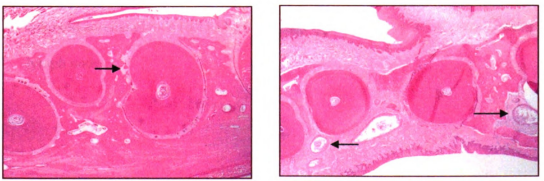
Figure 4-1. *Week One:* Oral squamous epithelial proliferation in mink exposed to PCB 126 for one week, and histological evidence at six months after the removal of the stimulus (PCB 126 diet). Tooth (T); Alveolar Bone (AB); Gingiva (Gin); Periodontal Ligament (PDL). Arrows on right point to squamous epithelial cysts that have invaded either the PDL and/or the AB. H&E



B) **PCB 126 Exposure for Two Weeks** (Figure 4-2). 1) *Weekly exposure*: One of the two mink that was euthanized following two weeks of dietary exposure to PCB 126 showed no histological evidence of lesion formation, while the other mink exhibited multiple cysts throughout the right maxilla deeming a rating of moderate. 2) *Six months post-exposure*: One mink died prior to the six months from wasting syndrome, therefore only having one month on clean feed following the two of PCB

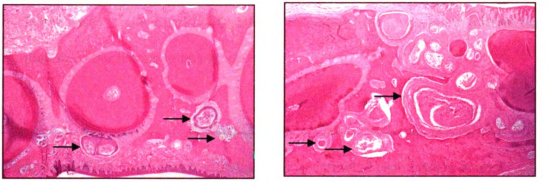
126 diet. However, this mink exhibited a severe lesion rating even without the grow-out period. Foci were multi-diffuse, and severe including invasive into the PDL. All quadrants were affected, and all apparent gingiva had been replaced with stratified squamous epithelial cysts. Periodontoclasia had become apparent during necropsy. The other mink that was euthanized at six months following removal of the contaminated diet after two weeks of exposure to the PCB 126 diet, exhibited multi-diffuse changes in the gingival tissue throughout all quadrants. Some increased space between incisors had become apparent. Because of the diffuse foci and clinical changes, a moderate rating was assigned to this mink.

Figure 4-2. *Week Two:* Histological evidence of oral squamous epithelial proliferation in mink exposed to PCB 126 for two weeks exposure, and six months after the removal of the stimulus (PCB 126 diet). H&E



C) **PCB 126 Exposure for Three Weeks** (Figure 4-3). 1) *Weekly exposure:* Both mink euthanized after three weeks of exposure to 24.0 μg PCB 126/kg feed had histological evidence of mild lesion formation. Squamous cysts were small and few in number in both mink. Some cysts had exfoliated centers, but no osteolysis was apparent. One mink appeared to have islands and/or cords that had developed in or near the PDL. 2) *Six months post-exposure*: One mink (T510) euthanized at six months post-three weeks PCB 126-exposure had a moderate lesion rating. The beginning clinical signs of the lesion were evident as spreading of the incisors, heavy plaque build up on the mandibular teeth, and histologically, several small foci dispersed throughout the gingiva (all quadrants), and. The jaw lesion was clinically apparent in another female (T560), as spreading of the maxillary incisors. Additionally, there was histological evidence of a severe lesion rating throughout the jaw, in which cystic formation was diffuse and multiple cysts were apparent in the PDL in this mink. This mink (T560) also exhibited signs of PCB toxicity, including enlarged liver (73.134 g), enlarged and discolored (mottled) kidneys, enlarged adrenal glands (0.1132 g), and highly vascularized and altered, lobular lymph nodes.

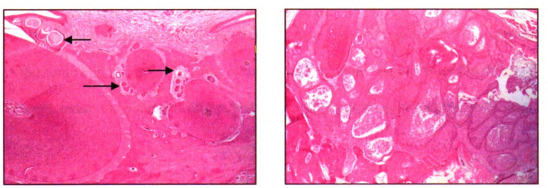
Figure 4-3. *Week Three:* Histological evidence of oral squamous epithelial proliferation in mink exposed to PCB 126 for three weeks, and six months after the removal of the stimulus (PCB 126 diet). Squamous epithelial cysts and/or nests depicted in the histology slide on the right are throughout the entire tissue. H&E



D) **PCB 126 Exposure for Four Weeks** (Figure 4-4). 1) *Weekly exposure:* Both mink that were exposed to the PCB diet for four weeks had mild or moderate histological evidence of the squamous epithelial cysts. One mink (T344) had mild changes only in the mandible. The other mink (T574) had minimal foci in the right maxilla, and no foci were observed in the left maxilla. Multiple diffuse foci were observed in the mandible, therefore warranting a rating of mild to moderate. 2) *Six months post-exposure:* Mink euthanized following four weeks on the PCB 126 diet and then six months on clean feed had histological evidence of a moderate of the lesion. The

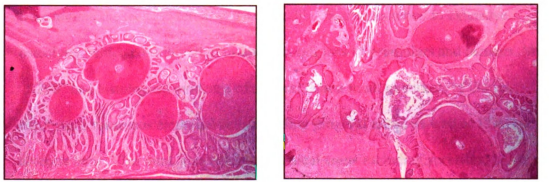
lesion was detected in all quadrants in both mink, but the severity did differ between quadrants in one mink. There was moderate to severe destruction to the gingival tissue, referring to replacement of gingival by stratified squamous epithelial cysts. In addition, the mink had clinical signs of displaced and loose teeth, as well as bleeding of the gingival tissue. One mink (T632) exhibited clinical signs of spreading of the incisors, as well as a heavy bacteria load evident by the plaque accrual. The other mink in this group (T586) also exhibited clinical signs of the lesion based on the mobility of teeth and receding of the gums, but to a lesser degree than the other female in this group.

Figure 4-4. *Week Four:* Invasive oral squamous epithelial proliferation in mink exposed to PCB 126 for four weeks, and six months after the removal of the stimulus (PCB 126 diet). In the histology slide on the right, there was no AB that remained in this section to support tooth structures, cysts and/or nests and cords destroyed and replaced almost all maxilla and mandible bone tissue. H&E



E) **PCB 126 Exposure for Five Weeks** (Figure 4-5). 1) *Weekly exposure:* Both mink euthanized at five weeks of exposure to the PCB 126 diet had histological evidence of the lesion, which was detectable in all quadrant of the jaw. The lesions in both mink were rated as severe, based on the diffuse nature and multiple foci throughout all the quadrants. The development of this lesion in one mink (T506) consisted mainly of cords, as opposed to cysts as in most of the mink. The lesion in the other mink (T552) was considered very severe. All gingival tissue and alveolar bone had been invaded and completely replaced by multiple stratified squamous epithelial cysts around dentition. Also, this mink had initial signs of interdental spreading of incisors. 2) *Six months post-exposure:* Six months following the five weeks on the PCB 126 diet, mink had severe diffuse lesions based on histological evidence of invasion of squamous epithelial cells throughout both the maxillae and mandibles. The mink exhibited clinical signs by loose and/or missing teeth as a result of loss of PDL attachment, osteolysis, and receding gums. Heavy plaque build-up was also noted in these mink, and is most likely a result of an increased bacterial load that is frequently observed in PCB 126 treated mink. One mink (T310) exhibited clinical signs of the lesion, including marked spreading of the incisors, increased plaque accrual, and visual thickening of gingival tissue. The other mink (T672) expressed the same clinical characteristics of the lesion including separation and mobility of the teeth, tartar and plaque build-up, and noticeable thickening of the gingival tissue.

Figure 4-5. *Week Five:* Development of oral squamous epithelial lesion with evidence of osteolysis in mink exposed to PCB 126 for five weeks, and six months after the removal of the stimulus (PCB 126 diet). Cysts and/or nests and cords have invaded the PDL of most of the teeth of this mink on the left. On the right, cysts and/or nests and cords have expanded and occupied almost all of the periodontium. H&E

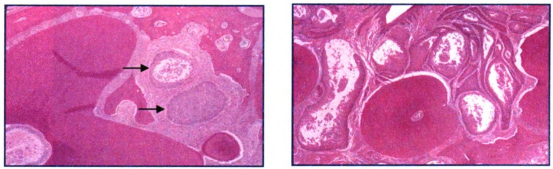


F) **PCB 126 Exposure for Six Weeks** (Figure 4-6). 1) *Weekly exposure:* The two PCB 126 treated mink (one 6-month-old and one 18-month old) that were euthanized following six weeks of dietary exposure to PCB 126 exhibited lesion development compared to controls. The adult mink expressed a mild lesion that consisted of small foci, somewhat diffuse. Infiltration into the PDL had not occurred, but some invasion into the alveolar bone was apparent. The yearling mink expressed a moderate lesion

that included infiltration of the PDL and invasion into the alveolar bone. Minimal periodontoclasia was observed in this mink. An additional mink (R42) that was euthanized at six weeks due to "wasting syndrome" already had moderately increased interdental spacing of the incisors and bloody gums; depleted adipose tissue and ascites were also observed during necropsy. 2) *Eight months post-exposure*: Clinically, one mink (T302) displayed extensive hyperplasia of gums on the inside of the lower palate and lips resulting in nodular growth formations (Figure 4-7). In addition, there was massive tissue destruction of the periodontal ligament and support around the incisors and cuspids (molars), as well as receding of the gums from around the teeth, externally. The mandible had succumbed to extensive osteolysis, which contributed to bi-lateral tooth loss of premolars and incisors. Mink "R290" had histological evidence of the oral lesion in all quadrants of the jaw, expressing squamous epithelial cells invading gingival tissue as well as PDL and alveolar bone. This mink also exhibited clinical signs including spreading of the incisors, and receding of the gums that made the canine teeth appear extended. Following necropsy, it was determined that the loss of PDL attachment, as well as receding gums and osteolysis around the teeth, caused the canines to both appear longer and actually protrude from the periodontal sockets. Another mink (R216) exhibited presence of the oral lesion by moderate histological changes that included multi-diffuse foci comprised of small islands of stratified squamous epithelium, as well as clinical changes observed as increased spreading of the incisors. The lesion rating in another mink (T624) in this exposure group was considered severe based on histological evaluation, as well as the observed clinical signs. In this mink, only two

maxillary incisors remained, and of the other remaining teeth (canines, premolars and molars), all of the teeth were very loose with minimal or no ligament or bony support remaining. This mink (T624) also had thickening of the gingiva of the soft and hard palate areas, but receding of the gingiva along the gum-line. Additionally, adverse effects of PCB toxicity were observed in this mink (T624) that included enlarged spleen (10.1258 g), enlarged liver (62.4531 g), and enlarged adrenal glands (0.1128 g). One mink that was exposed to PCB 126 for six weeks and then on clean feed for eight months had nodular growths above the oral palette. Histological examination indicated that these were potentially preneoplastic tumors, as they were an extension of the stratified squamous epithelial cells in combination with squamous cyst development originating from the gingiva. In one mink (T302), the tumor growth had developed on the dorsal side of the palate and measured 5.78 mm x 3.42 mm x 4.23 mm). Also noted was severe osteolysis of the nasal turbinates of mink in this treatment group that had extended far into the nasal cavity and frontal bone. This had also caused detachment of the nasal support structures. Osteolysis of the zygoma and zygomatic arches was also observed in this treatment group and is reported in Chapter 5. In addition, one mink in this group (R184) had an unusually brittle skull noted during necropsy.

Figure 4-6. *Week Six:* Evidence of an osteolytic oral squamous epithelial proliferation in mink exposed to PCB 126 for six weeks, and eight months following the removal of the stimulus (PCB 126 diet). The image on the right consists of teeth and lesion, all other structures have been destroyed and/or replaced by the lesion. H&E



Mink from Experiment 3 with histologically and clinically induced lesion:

T596 (70 days of exposure, or 10 weeks): Histologically, massive tissue invasion and destruction in the gingiva, PDL, and the alveolar bone was observed. The lesion was extensive, multi-diffuse with large and smaller cysts encompassing all visible tissues (except for teeth), thus appearing to be severe to very severe. Clinical observations revealed recession and deterioration of the gingival tissue along the gum line, resulting in the teeth and roots being exposed and unsupported. There was

Figure 4-7. Proliferation with substantial thickening of the gingival tissue forming nodular growths along the inside of the lower palate (along the dental arches), reaching almost to the central midline*. A) Nodular growths were observed in the mandible of a mink exposed to PCB 126 via the diet; B) the mandible from a control mink.

proliferation with substantial thickening of the gingival tissue forming nodular growths along the soft palate of the mandible (along inside of teeth), reaching almost to the center. All of the connective tissue along gums and the lips of the maxilla, as well as under nose and the nasal septum, was necrotic and detached from deterioration. Osteolysis was extensive throughout both the mandible and maxilla, and was noted into the nasal turbinates and frontal bones.

T600 (85 days of exposure, 12 weeks): Histologically, the lesion appeared similar to other mink with a severe to very severe rating. The mandible exhibited some proliferation forming nodular growths (Figure 4-7), however, not as extreme as the previous mink (T596). The mandible was literally "crumbling", with only the very distal section of the mandible remaining intact, and no other intact bone, and little gingival tissue remained. Because teeth had no remaining bone or tissue support, most had fallen out. The upper lip was still attached near the septum of the nose. However, the gums of the upper lip above the incisors were mostly necrotic.

T590x (86 days of exposure, 12 weeks): Grossly in this mink, tooth loss had occurred in the incisors, and premolars and molars were loose due to loss of PDL ligament support, osteolysis of alveolar bone and recession of the gums. Gums were bloody and swollen, and thickening of the gingiva was apparent along the dental arches of the mandible (Figure 4-7). Histologically, there was severe invasion of gingival tissue and the alveolar bone throughout the mandible and maxilla by nests and cords of squamous epithelial cells causing substantial tissue destruction.

T420 (140 days of exposure, 20 weeks): Grossly, gums frequently appeared bloody and swollen. Periodontoclasia occurred prior to loss of incisors. Two small nodules were observed in the upper palate cavity, consisting of stratified squamous epithelial cells in combination with squamous cyst development originating from the gingiva. In addition, one nodule was located in the right maxillary sinus, just off center of mid-line (location was 9.95 mm caudally of the canine), and measured 3.10 mm x 3.54 mm. The left maxillary nodule was located 16.08 mm caudally of the canine, and measured 3.23 mm x 4.10 mm. Histology of the nodules was similar to those reported in

the mink (T302) exposed to PCB 126 for six weeks and then on clean feed for eight months. The oral lesion was histologically classified as severe, with loss of alveolar bone due to invasion of squamous epithelial cells that caused osteolysis.

Results for PCB 126 analysis of mink livers:

Concentrations of PCB 126 in mink liver samples are presented in Table 4-1. The concentrations of PCB 126 in the liver tend to be elevated during exposure (consumption of the PCB 126 diet), and increasingly so with duration of exposure. These concentrations then noticeably decrease following the removal of the contaminated diet and animals are provided clean feed. As reported in Table 4-1, the longer the duration of exposure the higher the concentrations of PCB 126 in the livers of exposed mink. In addition, a gradual decrease in the PCB 126 concentration is observed in mink over time; the longer the mink have been provided a clean source of feed, the lower the liver concentration of PCB 126.

Two samples had higher lipid content than the rest of the samples (Table 4-1). Although lipid content could be associated with fatty liver syndrome following exposure to PCBs, artifacts due to the portion of the liver that is sampled for analysis could not be ruled out. The PCB 126 concentrations in the two samples with high lipid content were also high. One sample contained the highest concentration of PCB 126 although this individual mink had the lowest fat content in the liver. Another sample did not contain PCB 126 at the detection limit of 50 ng/kg on a wet weight basis. The weekly exposures and sacrifices evaluate PCB 126 accumulation in mink liver, and the

residual PCB 126 in the livers of the exposed mink can then be evaluate by the 6- or 8-month withdrawal data when the mink were on control feed.

Table 4-1. Liver analysis of percent lipid and PCB 126 concentrations were determined on a wet weight and lipid weight basis in livers from female mink (*Mustela vison*) exposed to 24.0 µg PCB 126/kg feed.

ID (Mink)	Exposure/ Withdrawal[1] *(Week/Month)*	% Lipid	PCB 126 (pg/g wet wt)	PCB 126 (pg/g lipid)
NA[2]	1/0	NA	NA	NA
T700[3]	2/0	17.1	28,990	169,600
T484 + T536	3/0	3.5	20,930	598,000
T574 + T344	4/0	10.9	100,500	922,300
T552 + T506	5/0	2.8	179,400	6,408,000
NA[2]	6/0	NA	NA	NA
	(Week/Month)			
T582x + T550	1/6	5.1	137	2,699
T602[4]	2/6	4.4	<50	<1,140
T560 + T510	3/6	4.9	805	16,430
T632 + T586	4/6	5.7	574	10,070
T672 + T310	5/6	3.8	1,635	43,030
R216 + R290	6/8	3.9	380	9,784
T624 + T302	6/8	5.2	455	8,742

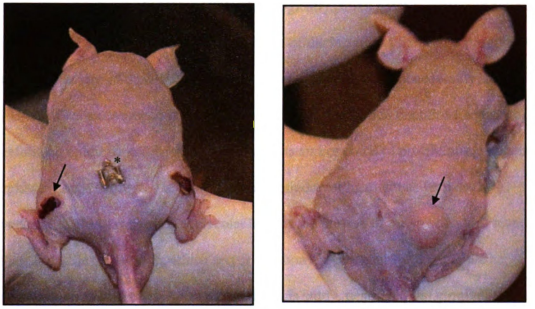
[1] Exposure is expressed in weeks, withdrawal is expressed in months.
[2] Data for Week 1 and Week 6 exposures are not available (NA).
[3] Mink T700 died on 2-13-03.
[4] Mink T602 was chronically on and off feed (refusal to eat), therefore ingesting an overall lesser concentration of PCB 126.

Experiment 3: Carcinogenic Study with Athymic Mice:

All mice developed lesions over the tissue implant sites within two to 12 days after implantation or injection of the cell suspension. The lesions initiated with redness of the skin, and what appeared to be an inflammatory response. These lesions generally healed within 10 days after formation, leaving signs of scar tissue. Two control mice died during the trial, that was not treatment related. All other mice remained healthy throughout the experiment.

Two mice developed measurable nodular growths at the transplant sites of the mink oral tissue. One mouse developed a round nodule that measured 5.11 mm x 4.92 mm x 2.92 mm within 14 weeks (98 days). The second mouse that developed a firm nodular growth received the gingival tissue from a different exposed mink, and the tumor measured 5.56 mm x 5.23 mm x 1.13 mm at 17 .5 weeks (122 days) after implantation of the tissue. A third mouse had a small growth develop, but was not measurable during the week it was noticed, or palpable at necropsy. This resulted in an incidence of 12% for tumor growth in mice that received gingival tissue from mink with probable SCC.

Figure 4-8. Nude, athymic mice implanted or injected with gingival tissue harvested from mink exposed to 24.0 μg PCB 126/kg feed via their diet. *Staple over incision.



A) Lesion B) Tumor

A) All mice developed a lesion over the transplant sites, including mice injected with the single cell suspension. It was determined that the lesion was caused by bacterial overload from the non-host oral mucosa. Thus, the viability of any potentially tumorigenic cells following the bacterial insult was questioned.

B) Two mice developed nodular growths at the transplant sites. These tumors were classified as non-malignant. However, they were identified as non-host (mink) squamous epithelial cells, and were considered extremely aggressive and robust.

DISCUSSION

Lesion Description:

The jaw lesion observed in the present study indicated that PCB 126 induced an aggressive form of oral SCC in mink. Histologically, small nests of stratified squamous epithelial cells characterized the initial stages of the lesion. As the lesion progressed, the number and size of these islands of cells generally increased. Subsequently, large islands of cells developed and formed cysts. These cysts often contained centers of exfoliated epithelia and possibly keratin. Nests and/or cords of proliferating squamous epithelial cells infiltrated into the adjacent periodontal ligament as well as the underlying alveolar bone. The alveolar bone is the portion of the maxilla and mandible that forms the dental arch, which serves as a bony investment for the teeth. The aggressive invasion of the cysts reported in this study, destroyed the gingival tissue and caused osteolysis of the alveolar bone. In general, osteolysis that is observed as loss of lamina dura, also known as the alveolar bone, tend to be aggressive malignant neoplasias that have a tendency to invade, expand, and perforate the cortex of the maxillae and mandibles (www.dentistry.vcu.edu). A benign lesion, on the other hand, would be more inclined to resemble compression, or even displacement, of the lamina dura (www.dentistry.vcu.edu).

In addition, clinical changes were observed in the condition of the gingiva and jaws (i.e. mandibles and maxillae) of PCB 126 exposed mink in the present study. Recession of the gingiva, destruction of the periodontal ligament and loss of alveolar bone support, are factors that were consistently observed with the oral lesion. These

conditions are characteristics of periodontoclasia, which results in the loosening of permanent teeth due to the breakdown and absorption of the supporting bone, and the teeth eventually fall out (Anderson et al. 1998). Additional changes included swollen, inflamed, and bleeding gingiva, and dental plaque buildup, which are predominantly external characteristics of severe periodontitis (Bartold et al. 2000). Heavy plaque build-up was also noted in PCB 126 treated mink, which is most likely the result of an increased bacterial load that was frequently observed in PCB 126 treated mink in this study.

A healthy periodontium, made up of four components including the gingiva, periodontal ligament, alveolar bone and cementum, is necessary to support and maintain the teeth and oral environment (Bartold et al. 2000). In general, gingival epithelia have important roles in the overall health of the oral environment, including protection against disease and cellular protection (in regard to nutrient balance, as well as toxicants) (Bartold et al. 2000). As observed in mink from this study, the overall health of the periodontium, including at least three of its four principal components (and possibly cementum, although it was not investigated in this study) has been adversely affected by dietary exposure to PCB 126, resulting in invasion and massive destruction of these tissues.

The lesion was histologically apparent following PCB 126 exposure as short as two weeks in mink on the PCB 126 diet. After this time interval, all animals exposed to the PCB 126 diet had evidences of the lesion. Week two mink expressed a lesion that was rated minimal to mild. Following week two, 100% of the remaining mink exposed to PCB 126 for weeks three through six expressed the lesion, with a lesion rating that

ranged from mild to moderate. Thus, as exposure time to the PCB 126 diet increased through week six, so did the severity of the lesion expressed histologically in the mink jaws. This indicated that the length of initial exposure to the chemical stimulus (PCB 126) does influence the length of time in which the lesion is detected histologically. Although mink in group "week one" did not have histologically evidence of the lesion at the week one necropsy, the mink in that same group that were grown out for six months on clean feed following that one week exposure did express the lesion histologically. In all of the weekly exposure groups, the lesion progressed in severity following the removal of the stimulus (PCB 126 diet). There was 100% incidence of the lesion in mink following six or eight months on clean feed, demonstrating that removal of the source does not allow the lesion to regress, but in fact the lesion continued to progress and worsen with no stimulus.

In mink that were exposed to PCB 126 for six weeks and then on clean feed for eight months, mink were giving watered-down feed because of periodontoclasia and/or aphagia. Nodular cysts above the oral palette were observed, and histological examination indicated the tumors to be an extension of the stratified squamous cells in combination with squamous cyst development. Also noted was severe osteolysis of the nasal turbinates of mink in this treatment group. This indicated the severity and destructiveness of this oral lesion as it progressed even at the duration of eight months post-exposure, suggesting that if these mink could naturally survive the toxicity of PCB 126 and the destruction of the lesion, malignancy and possibly metastasis might ensue.

Liver Analysis:

Analysis of liver PCB 126 concentrations from exposed mink indicated that when the PCB 126 feed was withdrawn, PCB 126 concentrations in the liver decreased over time. However, one could argue that the PCB 126 source had not been removed entirely due to the possibility of 1) the lipophilicity of PCBs, and/or 2) the high concentration that had accumulated in mink over the exposure period, which decreases slowly over time. In it possible that the mechanism(s) by which PCB 126 induces SCC acts through its physiochemical properties, i.e. lipophilicity, and thus, its bioaccumulation potential. However, based on the data presented in Table 4-1, the PCB 126 concentration in exposed mink decreased over time as depicted by the weekly exposure groups.

Nude, Athymic Mice:

The first group of mice that were transplanted with gingival tissue from the mink lesion developed lesions over the transplant sites. It was suspected that the lesions in the mice were caused by bacterial overload from the oral cavity of the mink, even though the tissue was treated with antibiotics. The antibiotic was changed to a broad-based spectrum penicillin and metronidazole combination to process the gingival tissues prior to implantation. In addition to the altered antibiotic combination, the gingival tissue was processed into a single cell suspension (pelleted cells) to eliminate or reduce the bacteria. This way the bacteria cells were exposed to the antibiotics as opposed to large tissue pieces on which only the surface was exposed. However, mice injected with the single cell suspension still developed the same lesions over the sites of injection.

Based on these caveats, the viability of any potentially tumorigenic cells was questioned. However, development of nodular growths despite the insult from the overload of oral bacteria and development of cutaneous lesions eluted to the potency of these gingival cells. In two mice that had the tissue transplants, nodular growths developed at the transplant sites were the lesions had been. These tumors were classified as non-malignant, but were identified as originating from non-host, foreign (mink) squamous epithelia. The results from this phase of the study were somewhat equivocal based on the limited number of mice in the study and the complications with mice developing lesions over the tissue sites. However, there are substantial implications to the growth of tumors, especially based on these complications with lesions in nude, athymic mice, even though growths were not classified as malignant. Therefore, the significance of these data cannot go unnoticed.

A study by the National Toxicology Program (NTP) in rats supports the results of SCC in mink from the present study, and supports previous indications that mink are more sensitive than rodents based on the incidence of confirmed lesions (NTP 1996*a*, 1996*b*). The NTP conducted a toxicologic and carcinogenic study with PCB 126 and TCDD in female Harlan Sprague-Dawley rats (exposure by gavage). PCB 126 showed clear evidence of carcinogenic activity based on the increased incidence of liver and lung neoplasias in rats (NTP 1996*b*). In addition, neoplastic lesions of the oral cavity, as gingival squamous cell carcinoma were observed in the oral mucosa of the rats, with an incidence of 0/53, 1/53, 1/53, 2/53, 2/53, 7/53, and 2/50 for doses of 0, 30, 100, 175, 300, 550, and 1000 ng PCB126/ kg BW, respectively. TCDD was found to cause non-neoplastic lesions of the oral cavity of rats, as gingival squamous hyperplasia in

generally the same percentage, although carcinogenic activity was not reportedly caused by TCDD (NTP 1996*a*).

However, an additional study from our laboratory was conducted to determine if the jaw lesion that was observed in mink could be induced in a sensitive strain of rats, the Long Evans (Aulerich et al. 2001). The weanling (28 days old) rats did not exhibit any evidence of the lesion grossly or histologically following 101 days of exposure to 20 or 100 μg of PCB 126, or 1.0 or 10 μg of TCDD via the diet, as was seen in mink exposed to 0.24 to 24.0 μg of PCB 126/kg feed.

Several characteristics of the lesion observed in mink from this study were consistent with criteria for malignant neoplasia, including increased proliferative activity, invasiveness of atypical cell type and diffuse infiltration, and tissue destruction. Morphological and behavioral characteristics of neoplasia can be used to differentiate between benign and malignant neoplasms (Cotran et al. 1999). However, a neoplasm can defy categorization, and ultimately, the morphological diagnosis of neoplasia is subjective, and based on criteria that are guidelines, not absolutes (Cotran et al. 1999).

Malignant neoplasms arising from soft tissues of the oral cavity often involve focal destruction of bone adjacent to teeth (Dennis 1991; McGlennon 1991; Myer 1994; Thompson and Pool, 2002). Osteolysis may be focal or diffuse, and may involve the mandible, maxilla, premaxilla, or hard palate (Dennis 1991). These oral malignancies adjacent to the alveolus tend to more lytic, whereas a benign process would tend to have a well-demarcated border that is reflective of its slow growth (Myer 1994).

Squamous cell carcinoma of the oral cavity in cats and dogs is an aggressive disease, which frequently invades the periosteum of the underlying alveolar bone in up

to 77% of canine cases (Thompson and Pool, 2002). Often the early lesions of gingival squamous cell carcinoma are relatively small nodular masses along the dental arcade. These nodules do not always form fleshy masses, and rarely metastasize to distal sites, usually at a rate of less than 3%. Lymphatic drainage from the gums is more restricted, and therefore limits potential for metastases (Head et al. 2002).

PCB-Induced Carcinogenesis - Pathways and Possible Mechanisms:

The definitions of genotoxic and epigenetic chemicals do vary somewhat, and it is recognized that some chemicals fit neither, and chemicals may in fact have attributes from both categories. However, there are apparent differences in chemicals that induce neoplasia, as well as their possible mechanisms of action (Klaunig and Kamendulis, 2001). PCB 126 may be considered a chemical carcinogen that has attributes from both categories. It does not require chronic exposure at threshold doses, it is irreversible, it may cause direct DNA damage, and it may have the potential to cause multi-organ tumorigenicity (genotoxic). It is unknown if PCB 126 exhibits species specificity because mink is a sensitive species. However, the study in rats by NTP demonstrates that PCB 126 causes multi-organ tumors in more than just one species, suggesting that PCB 126 is possibly a genotoxic agent. Reversibility was not observed following removal of the PCB 126 diet. In fact, the lesion continued to progress in all PCB 126-exposed mink. Therefore, a non-genotoxic classification that is reversible following removal of the test chemical stimulation does not seem accurate. In addition, epigenetic chemicals require chronic exposure and a threshold dose to elicit neoplasia (Klaunig and Kamendulis, 2001; Boatman et al. 2004). The existence of a threshold dose for PCB

126 has not been established, and chronic exposure is not required for the induction of SCC in mink.

Exposure to chemical carcinogens leading to the development of neoplasia can result from the imbalance between cell proliferation and apoptosis (Williams 2000; Boatman et al. 2004). Hyperplasia induced by nongenotoxic carcinogens is threshold-based and reversible following removal of the test chemical (Boatman et al. 2004). Chronic cell proliferation in preneoplastic cells, in turn, may result in papillomas and eventually leading to carcinoma (Boatman et al. 2004). However, the association between chemicals and carcinogenicity and cellular and molecular mechanisms, such as epidermal growth factor (EGF) receptors, tumor necrosis factor (TNF-α), interleukins, estrogen, and glucocorticoids, all appear to be mechanistic possibilities (Whysner and Williams, 1996; Klaunig and Kamendulis, 2001). Neoplasias developing in soft tissues (such as the gingiva) may have mutations that target oncogenes that regulate transcription factors or cell cycle proteins, causing this dysfunction or imbalance in cell proliferation (Cotran et al. 1999).

The PCB congeners that maintain a TCDD-like configuration apparently interact with the cytosolic protein, the aryl hydrocarbon receptor (AhR) (Chapter 1). Following activation and entry into the nucleus, and formation of the AhR-ARNT complex results in up-regulation of a battery of AhR-regulated genes (Rinaldi et al. 2002). At least six of the AhR-responsive genes are associated with carcinogen metabolism, including the potential carcinogen bioactivator CYP1A1 (Rinaldi et al. 2002). Due to the vigorous xenobiotic metabolism that occurs within the oral cavity (Rinaldi et al. 2002), AhR-activating agents, such as PCB 126, are characterized as potential intraoral carcinogens.

Bioactivation represents a valid strategy to reduce. CYP1A1 is one of the primary carcinogen-activating enzymes in oral mucosa that is suggested in initiation and promotion of oral SCC (Rinaldi et al. 2002).

The PCB 126-induced SCC mechanistic pathway responsible for its toxicity needs to be elucidated. The mechanism(s) by which PCB 126 induces SCC may be through the activation of transcription factors that we have not studied. A proposed epigenetic mechanism of carcinogenicity is cell cycling such as an increase in the cells entering the S-phase. A proliferating cell marker, Ki-67, can be used to determine the proliferative state, but we know there is an increase in cell proliferation in mink expressing the lesion. Additional analyses to investigate these pathways would include proliferating-cell-nuclear-antigen and immunohistochemistry. However, a study using the proliferative index and Ki-67 as markers of cell proliferation found no significant difference between dysplasia and carcinoma (Macluskey et al. 1999). Based on these results, it is suggested that epithelial proliferation may continue to increase during the transition from dysplasia to carcinoma, but this is likely to occur at a slow rate and Ki-67 expression is not a good indicator of neoplastic transformation (Macluskey et al. 1999).

Chemical carcinogens known to be tumor promoters have been shown to affect gap junctional intercellular communication (GJIC) structure and/or function and to inhibit cell-cell communication (Weis et al. 1998; Trosko et al. 2000). Dysfunctional GJIC plays a crucial role in the tumor promotion phase of carcinogenesis, which may involve the regulation of cellular growth, or more specifically down-regulation of GJIC (Weis et al. 1998; Trosko 2000). Inhibition of gap junction-mediated intercellular

communication has been proposed as a potential mechanism by which a wide variety of chemical and biological agents may act as promoters of neoplasia.

Species Comparisons:

Apparently, PCB 126 does not act similarly in mink as it does rats and mice. This may be due to species differences in aryl hydrocarbon receptor (AhR) characteristics, the target receptor for TCDD and dioxin-like compounds including PCB 126. Also, rodents may be less susceptible to the toxic effects of TCDD and similar compounds due to the rodents' ability to rapidly metabolize these chemicals (Huff et al. 1991). For example, the half-life of TCDD in rodents is between 12 and 30 days, depending on the rodent species (and strain). In contrast, the TCDD half-life in humans has been estimated at 7.1 years (Whysner and Williams, 1996).

Carcinogenicity, xenobiotic metabolism, as well as the responses that may be induced by exposure to chemicals, can be affected by species and strain differences, as well as inter-individual variation (Pelkonen and Nebert, 1982; Safe 1990). Additional conditions that affect the development or determination of a neoplasm in an animal include sex, contaminant, dose, duration (single, continuous, repeated, or withdrawal) and overall length of exposure/experiment (grow-out period or percentage of lifetime), targeted tissue(s) or complete organism evaluation, and complete neoplastic screening of tissues (Safe 1990).

The jaw lesion that was observed in mink exposed to PCB 126 in the current studies and earlier reports of TCDD appears to have some similarities to lesions reported in humans accidentally exposed to PCB contaminants, as well as marine

mammals inhabiting areas contaminated with high concentrations of organochlorine compounds. Following accidental PCB exposures in Japan through the consumption of contaminated rice oil (the *Yusho* incident), and Taiwan via the consumption of contaminated fish (the *Yu-Cheng* incident), a number of changes were noted in both the victims and their offspring. These changes included alveolar bone resorption, periodontal disease, oral pigmentation and increased depth of periodontal pockets (Shimizu et al. 1992; Hashiguchi et al. 1995). In addition, early eruption of teeth, gingival hyperplasia, spotted calcification of the parieto-occipital area of the skull, and poor skeletal development in human neonates were also reported as a result of exposure to these PCB contamination (Yamashita and Hayashi, 1985; Rogan et al. 1988). The lesions that resulted from humans exposed to PCBs through the ingestion of contaminated rice bran oil, the accidental spills of Yusho or Taiwan, is similar to the lesion reported in this study.

Beluga whales (*Delphinapterus leucas*) from the St. Lawrence Estuary have been reported as having a high incidence of unnatural pathology including unusual cancers and periodontitis with necrosis and loss of teeth, believed to be caused by exposure to environmental contaminants (Beland et al. 1993; DeGuise et al. 1995). Harbor seals (*Phoca vitulina*) and gray seals (*Halichoerus grypus*) from the Baltic Sea exhibited exostosis and osteolysis of the mandibular alveolar bone, as well as loss of teeth (Bergman et al. 1992; Mortenson et al. 1993). The pathology of the PCB 126/TCDD-induced proliferation of maxillary and mandibular squamous epithelia may be a lesion that is commonly occurring in a number of different species exposed to

environmentally relevant concentrations of contaminants suggesting that there may be an interspecies correlation.

Review of Carcinogenic Potential of PCB:

Cancers induced by PCB mixtures and TCDD have been focused primarily in the hepatic system, without mention of any oral carcinogenesis. The studies that have investigated the carcinogenic potential of PCBs have been with PCB mixtures except for one study that looked at the PCB congener 2,2',3,3',6,6'-hexachlorobiphenyl. It appears that these studies were based on constant exposure of the animals to the stimulus (the contaminant). Commercial PCBs have been reported to induce preneoplastic lesions and hepatocellular carcinomas in rats and mice when administered at concentrations of mg/kg generally per feed for extended periods of time (reviewed by Silberhorn et al. 1990). PCB mixtures that contained higher chlorine contents such as Kanechlor 500 and Aroclor 1260 induce neoplastic nodules and hepatocellular carcinomas more frequently. Studies have found that the liver is the primary target organ for PCB-induced tumorigenicity and carcinogenicity in rats and mice, although there is some evidence that the gastrointestinal tract may be affected (Silberhorn et al. 1990).

In regard to PCB-induced carcinogenicity, the U.S. Environmental Protection Agency (U.S. EPA) has concluded that there are sufficient data and evidence of the carcinogenicity of PCBs to animals. In addition, U.S. EPA holds a position on the toxicity of PCBs (commercial preparations in general), emphasizing the importance of analyzing all biological information as opposed to relying solely on tumor findings

(U.S. EPA 1996; Erickson 1997). The International Agency for Research on Cancer (IARC) has concluded that there is sufficient evidence that TCDD is a carcinogen in animals (Whysner and Williams, 1996).

CONCLUSIONS

This study was designed to evaluate the carcinogenic potential of a PCB 126-induced jaw lesion previously identified as mandibular and maxillary squamous epithelial proliferation. The invasiveness of squamous epithelial cysts and severity of osteolysis that resulted in periodontoclasia indicate that PCB 126 is a potent inducer of squamous cell carcinoma (SCC) in mink. Following only one week of exposure to PCB 126 and six months post-exposure on clean feed, SCC was histologically evident in mink. The present study met several criteria of neoplasia, including invasive behavior of atypical cells, tissue destruction and replacement with a new cell/tissue type, progression after stimulus removal, and tumor growth in non-host species. Also, individual variation appears to play a role in the susceptibility and the presentation of the lesion. Some family groups (siblings), even though they were assigned to different treatment groups, exhibited signs of the oral/jaw lesion more quickly and more severely that other mink. Although histological changes occur prior to clinical changes, clinical signs were observed visually prior to other changes. Thus, the value of this study in determining a minimal time necessary to induce the lesion histologically was important in identifying the onset of the clinical signs and progression of this lesion.

The accuracy of the histological classification of SCC in mink significantly contributes to the recognition of PCB 126 as a chemical carcinogen.

These results would suggest, to some extent that PCB 126 possesses characteristics that are consistent with attributes of a genotoxic carcinogen: it is irreversible, the lesion occurs at or near the site of exposure, it is potentially a complete carcinogen and probably causes direct DNA damage. However, PCB 126 may also possess attributes that are consistent with epigenetic carcinogens such as species specificity.

As aforementioned, there are apparent species differences, especially between mink and rodents, thus it is imperative when performing comparative studies to investigate all possibilities and data on these species differences especially when applying studies to other species, such as humans in a risk assessment situation. The U.S. EPA confirms that there are sufficient data and evidence of the carcinogenicity of PCBs (commercial mixtures) to animals (rodents). The results of the present study indicate that PCB 126 is carcinogenic by inducing oral squamous cell carcinoma in mink.

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CHAPTER 5

Bone Lysis Associated with PCB 126-Induced Neoplastic Oral Lesion of the Mandible and Maxilla in Mink (*Mustela vison*)

Bone Lysis Associated with PCB 126-Induced Neoplastic Oral Lesion of the Mandible and Maxilla in Mink (*Mustela vison*)

ABSTRACT

Polychlorinated biphenyls (PCBs) are ubiquitous contaminants in the environment that are resistant to biological and chemical degradation. The toxicity and bioaccumulation of PCBs give rise to serious and significant health effects. Because mink (*Mustela vison*) are sensitive to the toxicity of PCBs, the mink is an important animal model for studying 3,3',4,4',5-pentachlorobiphenyl (PCB 126)-induced effects on bone, as well as the neoplastic processes of PCB 126-induced oral squamous cell carcinoma (SCC) in the mandible and maxilla that causes aggressive osteolysis. Mink were fed a diet containing 24.0 μg PCB 126/kg feed for six weeks and then placed on clean feed to determine if PCB-induced oral SCC caused local (maxillary and mandibular) osteolysis, as well as systemic osteolysis. Radiographs were taken at weeks 0, 3, 6, 9, and 12 of the trial, and evaluation of radiographs included objective criteria (dental alignment, bone lysis, nail growth, and organ system evaluation) and seven skull measurements. Body condition was also assessed radiographically by measurement of body wall soft tissue, including subcutaneous fat. In addition, post-mortem skeletal mineral analyses of femurs were performed. Dental malalignment was observed as early as the third week of exposure in one treatment mink, and was apparent as incisor interdental spreading. Osteolysis was observed at six weeks of exposure in radiographs of four out of 10 treatment mink. Of the surviving mink at week 12, six out of seven PCB 126-exposed mink exhibited moderate spreading and/or movement of the teeth. No

malaligned teeth were observed in any of the control mink at any time, or had signs of osteolysis during the trial. The width of the zygomatic arch was significantly decreased between mink within the treated group over time. Investigation of the skulls revealed osteolysis had progressed up the maxilla and upward along the zygoma. The measurement of subcutaneous fat was significantly decreased in the mink receiving the PCB-treated diet as compared to the control mink. Thus, PCB 126 caused localized mandibular and maxillary osteolysis that aggressively progressed through the facial bones (i.e., the zygoma and nasal turbinates) until death ensued. However, the effects of PCB 126 were not manifested systemically on bone in mink.

INTRODUCTION

A number of neoplastic processes are associated with bone lysis. These mechanisms of osteolysis can include the replacement of normal bone cells with neoplastic cells (primary bone tumors), growth of adjacent soft tissue neoplasm into bone and/or pressure necrosis, tumor mass destruction of blood supply to bone, endocrine mediators, and paraneoplastic syndromes, such as hypercalcemia. Some of these neoplastic processes may be linked to the tumor-promoting activity and endocrine disruption of PCBs, demonstrating cancer potency and/or clear evidence of the carcinogenic potential of contaminants, including PCBs (Cogliano 1998).

Primary bone tumors (and secondary, such as metastatic carcinomas or sarcomas) develop when healthy bone cells are replaced with diseased cells, such as

facial and mandibular osteosarcomas, which can be characterized by osteolysis (Myer 1994; Whyte 1999; Thompson and Pool, 2002). Soft tissue tumors that are adjacent to bone can cause osteolysis by the cancer cells directly invading the bone by infiltration, or by causing erosion of the bone due to localized pressure (Rosenberg 1999; Thompson and Pool, 2002). Expanding tumor mass can cause ishemic necrosis of bone due to compression of vessels, which also is an important mechanism of osteolysis (Cramer et al. 1981). Neoplasms can alter endocrine homeostasis (Nelson and Feldman, 1991; Cotran et al. 1999), and can release mediators that cause a direct osteolytic effect through the local release of proteolytic enzymes by either tumor cells or host stromal cells, changing cellular function (Cramer et al. 1981; Body 1999; Cotran et al. 1999; Cockerell and Cooper, 2002). Paraneoplastic syndromes can often result in a systemic effect from neoplastic associated diseases, of which the most common are of endocrine origin (Cotran et al. 1999). Hypercalcemia, the most common of this syndrome, is frequently associated with malignant neoplasia as a result of invasion of bone (Dobson and Gorman, 1991; Cotran et al. 1999).

Neoplasia can be categorized as benign or malignant neoplasia, and are differentiated by morphological and behavioral characteristics. Benign tumors are non-cancerous, and are generally localized, slow growing, consist of well-differentiated cells, have limited potential for growth, do not invade surrounding tissue and do not metastasize to distant sites. Malignant neoplasms tend toward rapid growth, may contain anaplastic or poorly differentiated cells, characteristically invade surrounding tissue and can metastasize to other organ sites. Malignancies tend to be more aggressive and have a tendency to be more destructive than benign neoplasia, and if left untreated

may result in death (Myer 1994; Aiello 1998; Cotran et al. 1999; Cockerell and Cooper, 2002; Thompson and Pool, 2002). Invasiveness is a hallmark of malignant neoplasia, along with metastases, and it is the most reliable feature that differentiates malignant from benign tumors (Cotran et al. 1999).

It was hypothesized in this study that the oral squamous cell carcinoma induced by dietary PCB 126 caused systemic osteolysis, in addition to local (maxillary and mandibular) osteolysis in mink. To test this hypothesis, serial radiographs and post-mortem skeletal mineral analyses were performed in mink fed diets containing 24.0 μg PCB 126/kg feed.

METHODS

Five juvenile female (six months of age) mink and five adult (18 months of age) female mink were assigned to each of the two treatment groups (control and PCB 126-exposed) on day one of the trial. The exposed group of mink was fed a diet that contained 24.0 μg PCB 126/kg feed for the first six weeks of the 38-week trial. This group then received the standard ranch mink diet for the remaining 32 weeks of the trial. The other group, which served as the control group, was fed a standard ranch mink diet (Table 2-1). The standard ranch mink diet also served as the basal feed into which the supplemental PCB 126 was added. Complete methods of dietary mixing of PCB 126 are described in Chapter 2.

Mink were housed individually in wire-mesh cages (61 cm L x 76 cm W x 46 cm H) with a wooden nest box (30 cm L x 22.5 cm W x 25 cm H) attached to the outside of each cage at the Michigan State University (MSU) Experimental Fur Farm containment facility. Lighting in the room was maintained to simulate the natural light/dark cycle, Eastern Standard Time. Mink were observed daily for any signs of toxicity including decreased feed and/or water intake, refusal to eat, changes in physical appearance, activity level, and behavior alterations (Table 3-1). Mink were also weighed on day one of the trial, and body weights of mink were recorded on a weekly basis thereafter throughout the study. Food was provided fresh each day at approximately 140 g per female per day. Water was provided *ad libitum*.

Radiographs:

Radiographs of the mink were taken at the MSU Small Animal Veterinary Teaching Hospital. A 10:1 mixture of ketamine hydrochloride (25 mg/mL; Ketaset®, Fort Dodge Animal Health, Fort Dodge, IA) and Xylazine-20 (20 mg/mL; Butler®, Ben Venue Laboratories, Inc., Bedford, OH) was given at 0.4 mL/kg BW via intramuscular injection to anesthetize each mink for radiographs.

Two radiographic views of each mink were taken, which included the dorso-ventral (DV) view of the whole body and the lateral projection of the head. The radiographs were taken at weeks 0, 3, 6, 9, and 12 for all treatment animals and representative control animals. A tabletop technique with settings at 55 kVp, 2.5 mAs (400 mAs at 6.4 millisec) and 30 cm focal-film-distance was used (UltraVision® x-ray film/screen system, DuPont, Wilmington, DE). Evaluation criteria for radiographs

included subjective and empirical measurements of the skull and skeleton. Skull measurements are identified on Figure 5-1. Body condition was assessed radiographically by measurement of thoracic body wall soft tissues (Figure 5-7). This measurement included subcutaneous fat, and was taken on the right side of the animal, at the level of the ninth rib on the DV view of the radiograph. All measurements were acquired using a digital, sliding caliper (Digimatic Caliper, Mitutoyo, Japan) and recorded to the nearest 0.01 mm. One individual performed all measurements for consistency. Subjective evaluation of the radiographs including observations of alterations in bone opacity, dental alignment, and overall systemic bone condition was also conducted. In addition, nail growth and organ systems were evaluated from radiographs of the mink during the trial.

Necropsies:

At the end of the trial, mink were anesthetized with 0.4 mg/kg BW of the ketamine/xylazine mixture, and approximately 10 mL of blood were collected from each mink via cardiac puncture using a sterile 18 gauge needle, a 5 mL syringe, and a 10 mL serum collection tube. Blood samples were centrifuged (3000 x g, for 15 min), and aliquoted into 1.2 mL plastic cryogenic vials using disposable Pasteur pipettes. Serum aliquots were frozen at –74°C until analysis for osteocalcin.

Mink were euthanized with CO_2 gas following blood collection. A final body weight was taken and a gross examination was performed. Organs (adrenal glands, brain, heart, kidneys, liver, reproductive tract, spleen, and thyroid glands) were

examined, collected, weighed, and fixed in 10% buffered formalin (pH 7.4) for subsequent evaluations.

Osteocalcin Assay:

Serum samples were analyzed for osteocalcin (OC), an indicator of systemic bone formation, using an enzyme-linked-immunosorbent-assay (ELISA). The samples were analyzed for OC within 26 weeks of collection using an ELISA assay (Metra Osteocalcin EIA kit, Quidel Corp., San Diego, CA). Metra ^{TM} Osteocalcin assay is a competitive immunoassay that quantitatively measures intact (*de novo*) OC in serum, using a 96-well plate. Plates were read at 405-nm optical density on a Spectra Max 340 plate reader (Molecular Devices Corp., Sunnyvale, CA). Serum samples were run in duplicate following manufacturer's instructions.

Mineral Content Analysis of Femurs:

At termination of the study, femurs were removed and frozen. Bones were cleaned of all adhering tissue, weighed and length was measured prior to initial processing. Femurs were then tagged for identification, and extracted with ethyl ether using a Soxhlet apparatus for 24 hr. Bones were air dried under a hood for 72 hr and weighed to determine the dry fat-free weight. Femurs then were placed into acid-washed, porcelain crucibles and ashed in a muffle furnace (Thermolyne 30400, Barnstead/Thermalyne, Dubuque, IA) at 500°C for 16 hr. Ash was then weighed, and expressed as a percentage of the dry fat-free femur weight.

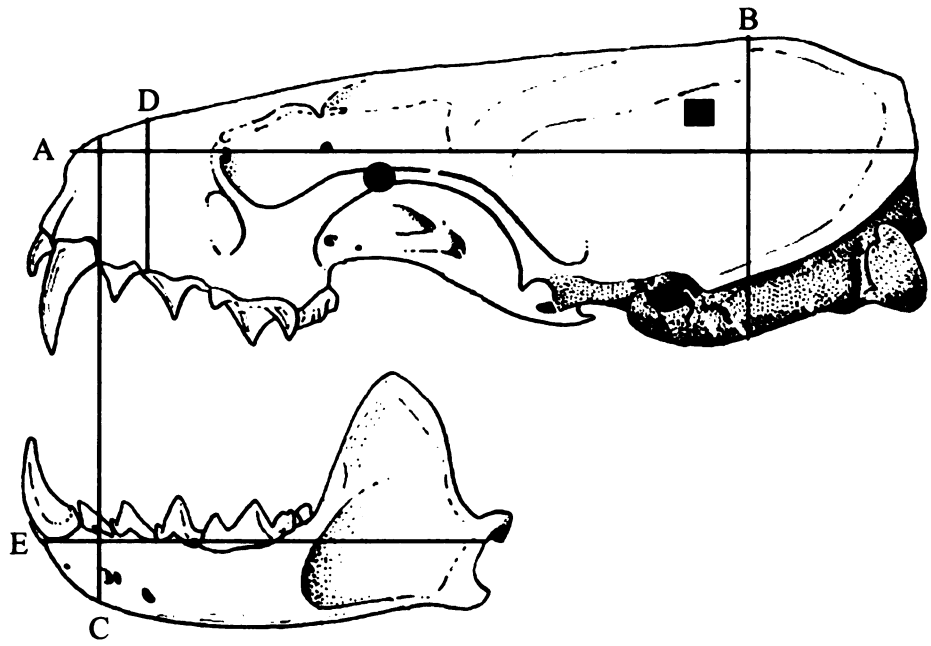
The ashed femurs were prepared for calcium and phosphorus analyses by nitric acid wet digestion. Samples were transferred to digestion vessels with two 5 mL rinses of 70% nitric acid (Omni Trace, EMD Chemicals, Inc., Gibbstown, NJ) and microwave digested (MARS 5, CEM Corp., Matthews, NC). Following digestion, 2 mL of 30% hydrogen peroxide (Sigma-Aldrich, St. Louis, MO) was added to the warm digest to complete sample oxidation. Digests were then transferred to volumetric flasks and brought to constant volume (50 mL) for analysis. Calcium concentration was determined by atomic absorption spectroscopy (Unicam 989, Thermo Electron Corp., Franklin, MA). Phosphorus was determined spectrophotometrically by the method of Gomori (1942).

Three skulls were retained intact from one PCB-exposed mink and one control mink at necropsy. These skulls were cleaned of all soft tissue by dermestid beetles for gross examination of the osseous tissue.

Statistical Analysis

Data were analyzed using Statistical Analysis System (SAS) program version 8.0 by one-way analysis of variance, and Tukey's method was used for adjustment of *p*-value in multiple tests. The mixed model procedure was used and the normality assumption was tested accordingly, and data was log transformed to meet the assumption of the mixed model. The level of significance was based on a Type I error rate of 0.05 ($p \leq 0.05$).

Figure 5-1. Skull measurements of the mink (*Mustela vison*) to evaluate PCB 126 induced osteolysis (left lateral view of the skull).



Footnote: A) Skull Length; B) Parietal Height; C) Nasal to Mandible Height (jaw was closed); D) Nasal to Palate; E) Mandible Length; F) Zygomatic Arch Width O (measured dorsally); G) Temporal Width ■ (measured dorsally).

Mink skull diagram was modified from W.J. Radke and R.B. Chiasson (1998) and Beckett, unpublished drawings.

RESULTS

Objective Criteria:

Dental malalignment (Figure 5-2) was observed on the second set of radiographs taken at three weeks of PCB 126 exposure in one animal (R42) of 10 PCB 126 exposed mink, in which the left maxillary incisor interdental spaces were increased. At six weeks of PCB 126 exposure, an additional five animals (totaling six out of 10) on treatment had mild changes in dentition alignment that included spreading of maxillary incisors and incisor unevenness. One animal had increased interdental space between the incisors, as well as a possible decrease in the enamel layer of the incisors. Radiographs taken during the 12^{th} week of the trial (six weeks post-PCB exposure) revealed further dental malalignment in five of the remaining six exposed mink. In mink that had previously exhibited interdental spreading of the incisors, radiographs displayed progressive increases in the spreading of incisors and the dental malalignment (Figure 5-3). Three of these mink had considerably increased interdental spacing of the incisors that was categorized at a moderate level.

Jaw osteolysis was observed at six weeks of PCB-exposure in four animals out of the 10 mink receiving the PCB-treated diet, with radiographic changes consisting of loss of the maxillary and mandibular lamina dura around the canines and incisors, and increased lucency around the canine tooth roots. At nine weeks (three weeks post-PCB 126 exposure) radiographs showed that the degree of osteolysis had increased in all of the mink that had previously expressed lysis. At 12 weeks (six weeks post-PCB 126 exposure), all treated mink had progressive maxillary osteolysis that ranged from mild

to moderate. A progressive and marked increase in maxillary and mandibular osteolysis that was considered severe in the exposed mink was observed at week 15 of the study (nine weeks post-PCB 126 exposure) in the radiographs.

Intact Skull:

Osteolysis was observed on the intact skulls that were cleaned by dermestid beetles and preserved (Figures 5-4 and 5-5). The destruction of bone had penetrated the hard palate and progressed into the nasal turbinates, the frontal lobes, and the zygomatic arches.

Radiographic Measurements (Skull and Subcutaneous Fat):

The overall change in width of the zygomatic arch significantly decreased (p = 0.03) within the PCB 126 treatment group over time (treatment-by-day effect) as measured on the sets of radiographs (Table 5-1). The zygomatic arch width within the control group did not change, and this measurement was not significantly different between control mink and mink exposed to PCB 126 via their diet. Osteolysis of the zygomatic arch, as well as the zygoma, was observed in the skulls cleaned by the dermestid beetles (Figure 5-6). No other significant differences were observed in skull measurements in the PCB 126-exposed mink (i.e. skull length, parietal height, nasal to mandible height, nasal to palate height, mandible length, or temporal width).

Subcutaneous Fat:

Subcutaneous fat was significantly decreased ($p = 0.03$) in the PCB 126-exposed mink as compared to the control mink (Figure 5-7). The subcutaneous fat measured in the mink within the PCB 126 treatment group had significantly decreased ($p = 0.0004$) over the 15-week radiographic series, (as was statistically measured as the treatment-by-day effect).

Skeletal Changes:

No skeletal malformations or abnormalities, including osteolysis of long bones or bone fractures, were observed in any of the mink, at any of the time points in the radiographs.

Osteocalcin:

The bone formation marker, osteocalcin, which was analyzed in serum, did not appear to show any cross-reactivity of the antibody with mink. Therefore, no data was obtained from this assay.

Mineral Analyses of Femur Bones:

There were no significant differences in the parameters measured for the mink femurs between the control mink and the mink receiving 24.0 μg PCB126/kg feed. Weight of femurs after bone ashing was not significantly different between the two groups of mink with an average group weight of 0.74 ± 0.05 g and 0.66 ± 0.04 g for control and PCB-exposed groups, respectively. Mean calcium content was 245.16 ±

4.97 and 245.95 ± 4 mg Ca^{+} /g bone for control and PCB 126 group, respectively. Mean phosphorus content of 107.94 ± 1.79 and 106.64 ± 1.46 mg P /g bone, for control and PCB 126 exposed mink, respectively, which were not statistically significantly different.

Other Pathologic Changes:

Nail Growth Abnormalities: No abnormal growth of the toenails was detected on any of the sets of radiographs. Increased toenail growth was observed visually during daily observations in one mink exposed to PCB 126 at 16 weeks, and in several animals from this same exposure group by 20 weeks, but only in the front feet. However, nails were not histologically examined in this study.

Kidney Uroliths: Uroliths were detected in one control animal in week 12 of the radiographic series. No other mink on trial, either exposed to PCB 126 or in the control group, had any signs of urinary calculi.

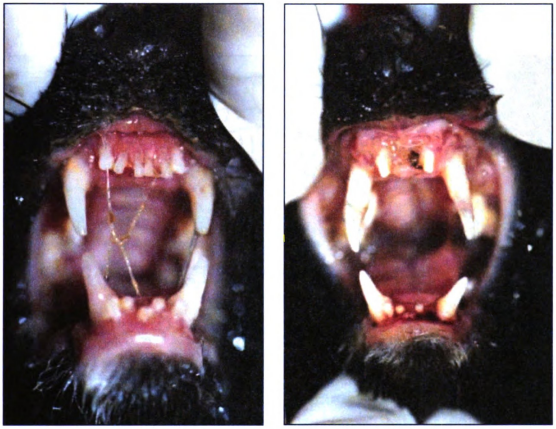
Organ Changes: Other conditions that were observed on the radiographs included cardiomyopathy (T402) and enlarged aortic arch (T600) that appeared normal thereafter. These cardiac conditions were observed on the initial set of radiographs, suggesting a pre-existing condition in these two animals. Cardiomegaly (R216) and microcardia (T402) (the initial radiograph taken of T402 showed cardiomyopathy) were observed at 12 weeks of the study. Both of these mink were receiving the PCB 126 diet.

DISCUSSION

Localized bone resorption of the maxillae and mandibles of female mink was due to direct invasion of gingival squamous cell carcinoma (SCC) induced by consumption of PCB 126 mixed into the diet at a concentration of 24.0 μg PCB 126/kg feed. With the extent of the destruction of the lesion in the oral cavity, including the gingiva, mandible and maxilla, assessing the aggression of the lesion in bone was important in understanding the effects of PCB 126 and other contaminants and the potential systemic effects they may have.

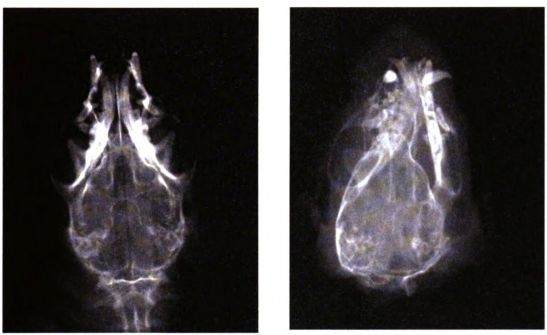
The dental malalignment expressed in one animal occurred as early as three weeks of exposure to the PCB 126 diet. In subsequent radiographs of this same animal, the incisors appeared to be very thin suggesting a decrease in the enamel layer, making them appear to be floating in the maxilla. This has been explained by the destruction of the lamina dura, which can cause the teeth to appear as if they are floating in the

Figure 5-2. Dental malalignment and increased interdental spacing in mink induced by consumption of a diet containing 24.0 μg PCB 126/kg feed. A) Notice inflammation of gingival tissue and moderate spreading of incisors. B) Canines appear extended and incisors were missing (mandibular and maxillary); gums were bloody and swollen.



A) B)

Figure 5-3. Radiographic images of female mink (*Mustela vison*) showing dental malalignment and increased interdental spacing induced by dietary exposure to 24.0 µg PCB 126/kg feed. A control mink (left) depicts normal lucency in jaws and alignment of teeth compared to a PCB 126 exposed mink (right), at Week 12 (6-week PCB-exposure period followed by six weeks of being on control feed).



Control PCB 126

Figure 5-4. Extensive osteolysis of mandibular and maxillary alveolar bone in a female mink (*Mustela vison*) exposed to 24 µg PCB 126 /kg feed. Note: no bony support surrounding dentition, resulting in loss of teeth. (Skull cleaned by dermestid beetles.)

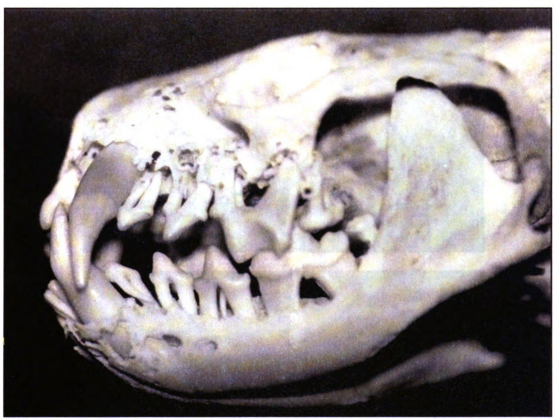


Figure 5-5. Bone lysis and tooth loss observed in the mandible, maxilla, and nasal bones in mink exposed to 24.0 μg PCB 126/kg feed. (Skulls cleaned by dermestid beetles.)

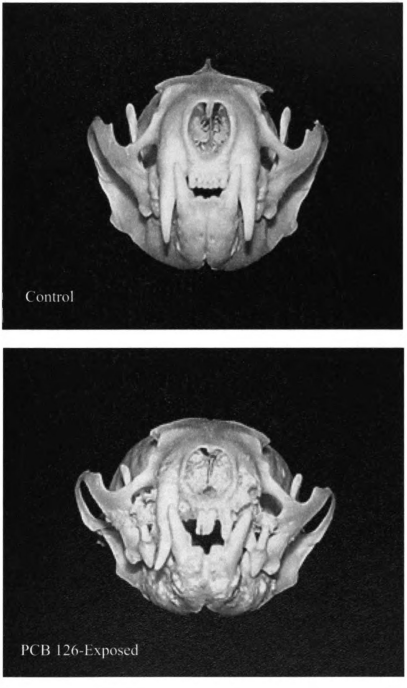


Table 5-1. Measurements (mm) of skull and subcutaneous fat of female mink (*Mustela vison*) exposed to PCB 126 through their diet (24.0 µg PCB 126/kg feed). [1, 2]

Parameter (mm)	Tri-Weekly Radiographic Series For PCB 126 Exposed Mink					Overall Mean ± SE for Trial	
	1	2	3	4	5	Treatment	
	(Week 0)*	(Week 3)*	(Week 6)*	(Week 12)*	(Week 15)*	Control	PCB 126
Skull Length	56.76 ± 0.47	57.26 ± 0.49	57.69 ± 0.49	58.79 ± 0.60	57.40 ± 0.57	57.55 ± 0.75	57.58 ± 0.52
Parietal Height	23.51± 0.27	23.60 ± 0.29	23.27 ± 0.27	23.46 ± 0.37	23.81 ± 0.35	24.13 ± 0.45	23.53 ± 0.31
Nasal + Mandible	25.20 ± 0.29	24.52 ± 0.31	24.51 ± 0.29	24.61 ± 0.41	23.91 ± 0.36	24.56 ± 0.48	24.55 ± 0.33
Nasal to Palette	13.30 ± 0.20	12.62 ± 0.22	13.41 ± 0.20	13.32 ± 0.28	12.84 ± 0.26	12.94 ± 0.34	13.10 ± 0.23
Mandible Length	33.84 ± 0.42	32.35 ± 0.45	32.37 ± 0.42	32.30 ± 0.56	31.11 ± 0.53	32.41 ± 0.70	32.40 ± 0.48
Zygomatic Arch	34.78 ± 0.27[a]	34.55 ± 0.28[a]	33.88 ± 0.27[a]	34.14 ± 0.33[a]	33.45 ± 0.32[b]	34.35 ± 0.42	34.16 ± 0.29
Temporal Width	27.99 ± 0.25	27.74 ± 0.26	26.88 ± 0.24	26.94 ± 0.31	26.79 ± 0.29	27.42 ± 0.39	27.27 ± 0.27
Subcutaneous Fat	10.40 ± 0.81[a]	9.61 ± 0.85[a]	7.14 ± 0.79[a]	7.08 ± 1.0[b]	9.10 ± 0.96[a]	10.95 ± 1.27	8.67 ± 0.88

[1] Data are presented as means ± standard error (SE) of the mean.

[2] Means in the same row with different letter superscript are significantly different ($p \leq 0.05$).

* Week refers to week on trial, with a 6-week exposure period followed by removal of the PCB 126 diet for a 8-month period on the control diet.

Figure 5-6. Osteolysis of the zygoma and zygomatic arch in a female mink (*Mustela vison*) exposed to a diet that contained 24.0 μg PCB126/kg feed.

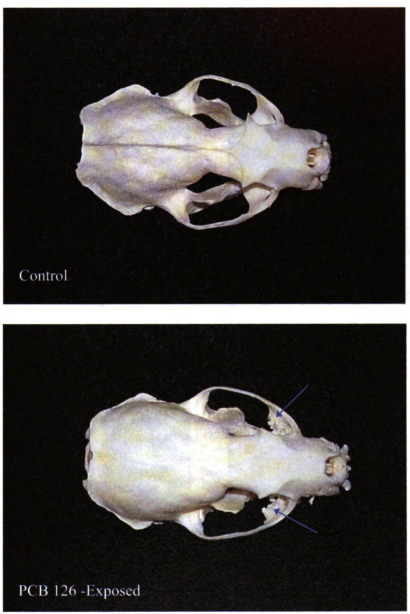
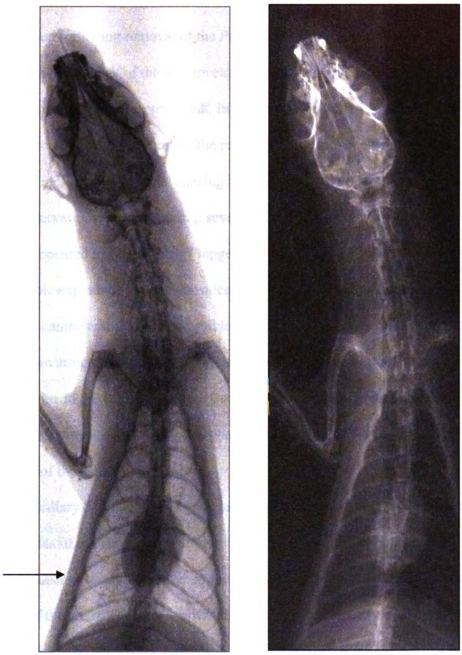


Figure 5-7. Radiographic image of a female mink (*Mustela vison*) exposed to 24 μg PCB 126 /kg feed via the diet for six weeks, and then on control feed for three weeks. Radiograph depicts decreased subcutaneous fat (arrow), and increased interdental spacing of the incisors, as well as increased lucency in the jawbones.



*Same radiograph (of the same mink) shown in positive and negative film exposure.

demineralized maxilla and mandible, thus allowing teeth to become displaced (Myer 1994).

The number of affected mink and the severity of the dental malalignment continued to increase as the trial progressed, and led to periodontoclasia. Tooth loosening and displacement varied from subtle to complete tooth loss over the course of the trial, even following removal of the PCB 126 after week six. The radiographs taken during the twelfth week of the trial revealed further dental malalignment in exposed mink as compared to the control mink. In several of the PCB 126 treated mink, bone support was completely lost around the roots of one or more of the canine teeth in addition to increased interdental spacing of the incisors that was observed. During regular observations and monitoring, several of the treatment mink displayed canine teeth that appeared to be "growing" longer, or extended from previous observations. Two possible explanations were considered for this observed change in canine length. Firstly, the canine could have appeared longer due to the recession of the gums or gingiva from around the base of the tooth. Secondly, this apparent lengthening could have been due to the loss of connective support of the teeth, including the loss of periodontal ligament and the alveolar bone, that allowed the tooth to actually loosen and extend out of the socket.

Maxillary and mandibular lysis in the mink was first observed in the radiographs as a mild loss of the lamina dura around the canines and incisors of the maxilla, and also increased lucency around the roots of the canines that eventually led to increased mobility of the teeth. This lucency was detected in mink as early as six weeks following exposure to the PCB 126 diet. Osteolysis around the teeth was observed as both focal

and diffuse in the mink jaws. However, as the trial continued, the loss of osseous tissue became diffuse through the jaws. Bone resorption became so severe, that no bony support remained around the incisors, canines, or the premolars in some of the mink that were exposed to the PCB126 diet (Figure 5-4).

Four tissue structures comprising the periodontium, the periodontal ligament, gingiva, alveolar bone, and cementum (Jones and Boyde, 1999) appear to be adversely affected in mink exposed to PCB 126. Receding gingiva, loss of periodontal ligament, and loss of alveolar bone support were factors that contributed to the spreading and malalignment of teeth noted in the radiographs in this study.

Oral gingiva and the periodontal ligament appear to be adversely affected by invasion of squamous epithelial cells prior to osteolysis of the lamina dura. Thus, tooth support is decreased by the loss of fibrous connective tissue provided by the periodontal ligament, as well as the surrounding gingival tissue. However, in the radiographs, lucency around teeth was observed prior to the gingival and periodontal effects. The loss of tooth support from the periodontal ligament, the gingival tissue, and alveolar bone resulted in the teeth spreading, loosening, and becoming displaced, and often falling out. Previous studies by Render et al. (2000*a*, 2000*b*, 2001) originally described the oral lesion induced by dietary exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and 3,3',4,4',5-pentachlorobiphenyl (PCB 126) as squamous epithelial proliferation. Dental clumping and mandibular and maxillary osteolysis in mink kits exposed to PCB 126, including loose and displaced dentition following the PCB 126 exposure for one to two months. In addition, the mink kits had nodular proliferation and gingival thickening of the oral mucosa (Render et al. 2000*b*).

Because osteolysis of the lamina dura was observed, this indicated a malignant neoplasm, as opposed to a benign lesion that would more resemble compression, and/or displacement of the lamina dura (Rosenberg 1999; Thompson and Pool, 2002; www.dentistry.vcu.edu). In addition, aggressive malignant neoplasms have a tendency to invade, expand, and perforate the cortex of the maxillae and mandibles (www.dentistry.vcu.edu). The destruction and aggressiveness of this oral SCC caused partial lysis of the cortex of the maxillae and mandibles, as well as partial to complete lysis of several tooth sockets. Bone lysis and dental displacement continued to worsen following removal of the PCB 126 stimulus. In mink from this study that exhibited severe oral neoplasia and osteolysis, the osteolysis progressed into the nasal turbinates and caused destruction throughout the nasal passage and frontal bones over time.

The width of the zygomatic arch significantly decreased during the course of the trial in PCB 126 exposed mink within the treatment group. The aggressive osteolysis affecting the maxilla had progressed upward into the zygoma, compromising its structural integrity and allowing the zygomatic arches to deviate axially, or fold in upon itself. This effect may also be related to, or explain, the visual effects of flattening and broadening of skulls that were noted in a prior trial of mink exposed to 24.0 μg PCB 126/kg feed.

No other skull measurements were significantly different between control and treatment mink, or within the treatment group over time. However, in a study by Allen and Leamy (2001), the authors used fluctuating asymmetry (FA) to assess the effects of low doses of TCDD on size and shape of the mandibles in offspring from adult mice exposed during pregnancy. The results from that study showed that TCDD decreased

the size of mandibles in mice. The largest effect was on the mandible landmark in close proximity to the teeth, particularly the molar tooth row area of the mandible. Allen and Leamy (2001) suggest that FA has become an important diagnostic tool in areas such as environment and conservation biology, and ecotoxicology. However, based on the results from the current study with mink, skull measurements were not affected by exposure to PCB 126, with the exception of zygomatic arch width, which was the result of osteolytic changes.

The results from various mineral analyses such as percent bone ash, and femur Ca and P concentrations indicated that there were no differences between control mink and mink that received the diet containing PCB 126. These data, in addition to the fact that there were no observed changes on the radiographs, indicated that a systemic effect on bone resorption was not occurring in mink consuming the PCB 126 diet. Contrary to our study, Lind et al. (1999) demonstrated pronounced effects in rats by PCB 126 that resulted in decreased length of tibiae, as well as increased bone mineral density and cortical thickness. Exposure to PCB 126 impaired the mineralization process of tibiae as indicated by significant increases in organic content and in osteoid surface (Lind et al. 1999). In another exposure study, Andrews et al. (1989) reported that commercial PCBs (Aroclor 1254) caused changes in the structural morphometry of the femur in young, growing rats. Femur weight, volume, and length were significantly decreased, and femur density was significantly increased in rats exposed to 25 mg/kg Aroclor 1254 (Andrews et al. 1989). Jamsa et al. (2001) reported TCDD-induced inhibition of tibial growth in Han/Wister rats at a concentration of 170 μg/kg BW, and the same effect observed in Long-Evans rats at a concentration of 1.7 μg/kg BW.

Although osteocalcin (OC) was analyzed in this study, the antibody did not appear to show cross-reactivity in mink. Based on the use of the OC assay in various carnivorous species, the ELISA kit was believed to show positive cross-reactivity of the antibody with mink protein. Osteocalcin (bone Gla protein) is one of the principal non-collagen proteins found in bone and dentin (Gundberg and Nishimoto, 1999; Jones and Boyde, 1999), and contains γ–carboxyglutamate residues that bind to hydroxyapatite (Gundberg and Nishimoto, 1999). Osteocalcin is secreted by osteoblasts (bone-forming cells), odontoblasts, and hypertrophic chondrocytes and is therefore known as a bone formation marker. Decreases in OC concentrations in serum have been reported as a result of dietary exposure to PCB 126 (Lind et al, 2000). Although no data were obtained for this study, OC has proved to be a valuable marker in bone research (Body 1999; Gundberg and Nishimoto, 1999).

Osteolysis affecting the skulls and jaws of other wildlife species has been reported, but not frequently. A disease complex of the skull reported in beluga whales (*Delphinapterus leucas*) (Beland et al. 1993) and Baltic grey seals (*Halichoerus grypus*) (Bergman et al. 1992; Lind et al. 2003) may be a similar lesion to that observed in laboratory studies with mink (Render et al. 2000*a*, 2000*b*, 2001), and now reported in wild populations of mink (Beckett et al. 2005). Similar to the lesion reported in mink, characteristics of the skull disease complex reported in the Baltic seals and belugas included loss of alveolar bone, widening of the teeth sockets, tooth loss, osteolysis, and frequent severe erosions and perforations of masticatory bones. High PCB and DDT concentrations in belugas from the St. Lawrence Estuary (Beland et al. 1993) and high organochlorine compounds in Baltic seals from the Baltic Sea (Lind et al. 2003) may

have contributed to the lesions that developed into the skull disease complex. A skull of a mountain sheep exhibited some characteristics that appear similar to the lesion presented in this study (Bleich et al. 1990). In addition to lateral asymmetry of the cranium and rostrum, the skull exhibited areas of osteoporosis in the mandible and the alveolar process of the maxilla, as well as malocclusion of the molars (Bleich et al. 1990). However, the authors eventually concluded that the lesion was caused by malnutritional osteoporosis.

Following accidental PCB exposures in Yusho, Japan and Taiwan, some human newborns of victims that ingested PCB contaminated rice oil were noted to have poor skeletal development (Yamashita and Hayashi, 1985; Rogan et al. 1988). These skeletal changes in neonates included spotted calcification of the parieto-occipital area of the skull, early eruption of teeth, and gingival hyperplasia (Yamashita and Hayashi, 1985; Rogan et al. 1988). These skeletal and oral changes in humans that were caused by the PCB exposure suggest some similarity to the neoplastic lesion that is being reported in mink.

Body weights of the female mink were taken weekly, and the significant differences in measurements of subcutaneous fat that were observed on the radiographs corresponded with loss in body weights that were recorded during the same three-week intervals (data not shown). As seen in other studies with mink, animals exposed to this class of compounds experience significant weight loss, often termed "wasting syndrome", that may not be entirely due to a decrease in food consumption. The weight loss observed in the PCB-exposed mink may have been complicated by conditions of neoplastic disease as well.

Five female mink from the PCB treatment group expressed classic signs of cachexia, which is the indirect result of malignant neoplasia. Animals often exhibit anorexic, generalized wasting, and overall weakness (Cotran et al. 1999). Several of these PCB exposed females either died or were euthanized during week six of the trial, and others were euthanized between the 9^{th} and 12^{th} weeks of the trial, due to their poor condition and deteriorating health. The loss of these females may account for the statistical increase reported for the radiographic measurement of subcutaneous fat that was taken during the 12^{th} week of the trial (Table 5-1). In addition, several of the mink that expressed the lesion also exhibited aphagia. This resulted from the presence of the lesion, and was due to gums that were swollen and often bloody, and the mobility of the teeth. Often the osteolysis was so extensive throughout the jaw that several of the PCB exposed mink exhibited the loss of teeth. Mink that expressed any difficulty in eating were offered additional feed that had been watered down to minimize their need to chew food.

Neoplastic Bone Lysis:

The bone lysis reported in the present study may have occurred as a result of a type of soft tissue neoplasia that caused a direct effect on the adjacent bone. Histologically, lysis occurred as a result of invasion of the bone by the cancer cells. Alternatively, neoplasms can also release mediators that cause a direct osteolytic effect through the local release of proteolytic enzymes by either tumor cells or host stromal cells, or both, to break barriers such as basement membranes, cell-cell junctions, and the extra-cellular matrix (Cramer et al. 1981; Body 1999; Cockerell and Cooper, 2002).

Bone resorption can be affected by neoplastic cells through mediators of osteoclast-activity, causing an increase in the stimulation and proliferation of osteoclasts (the bone cells responsible for resorption) (Body 1999). Osteoclast-activating factor can be mediated by either tumor cells or normal host cells that act systemically and distant to the tumor (Sasaki et al. 1998; Cockerell and Cooper, 2002). One of the primary cellular mechanisms of osteolysis associated with metastatic cancer may be osteoclast-mediated (Sasaki 1998).

A form of paraneoplastic syndrome may be occurring in mink exposed to PCB 126 in this study. However, in mink observed with the bone lysis and oral lesion, the duration between lesion progression and the demise of the mink was rapid and thus, prohibiting systemic changes. Hypercalcemia, the most common paraneoplastic syndrome, is frequently associated with malignant neoplasia, including squamous carcinoma, and results from the invasion of bone (Dobson and Gorman, 1991).

The mink exposed to PCB 126 in this study may have succumbed to death before the cancerous state of the lesion had spread systemically. Neoplastic diseases are reported less frequently in mink. This is because mink are generally maintained on commercial fur farms, and pelted before they reach old age. However, because mink and ferrets (*Mustela putorius furo*) are both mustelids and closely related, one would expect the potential for a similar occurrence in tumor rate. In the domestic ferrets, lymphoma is the most common type of neoplasm encountered, with both solid and leukemia types being reported (Williams 1991). Therefore, if the mink in this study had survived to old age it is possible that the neoplastic lesion would have progressed systemically.

The specific mechanism of action of PCBs or TCDD on bone has not been fully elucidated. There may be more than one mechanism, and potentially a complex of integrated pathways that is required for specific effects on bone, either locally or systemically. Species differences may exist in the mechanism(s) of action responsible for the bone lysis observed between mink in the present study and rats and mice from studies referenced herein. Also, rodents may be less susceptible to the toxic effects of PCBs and TCDD due the rodents' ability to rapidly metabolize the chemicals (Huff et al. 1991). Because the mechanism inducing this neoplastic lesion in mink in this study has not been elucidated, it is suggestive that PCB 126 may interact through several different and complex pathways, thus altering or interacting with the endocrine system.

Other Changes:

Orthokeratotic hyperkeratosis of the toenails occurred in mink exposed to PCB 126 over the length of the study. Radiographs did not reveal any osteolytic changes in the nails. However, clinical changes of the nails, including marked thickening, elongation, and deformity were observed during daily monitoring. Only the front nails had abnormal growth, and nails on the hind feet appeared normal. Minimal growth in the nails was first noticed at approximately 16 weeks of the trail in mink exposed in the PCB 126 diet. The observed growth in nails varied between mink in the PCB 126-exposure group. Although two mink exposed to PCB 126 had minimal changes in nail growth, two other mink in this same group had extreme nail growth that continued until

termination of the project. The nail growth in these two mink often hindering their mobility to move around their cages, because the nails would get caught (hooked) on the wires.

The one mink that radiographically displayed uroliths, or stones, was a control mink that had not previously been a part of the selected control group for films. Most likely, based on the size of the stones, this was a pre-existing condition prior to the start of this study. No other calculi were observed during this trial. Thus, it indicates that PCB 126 does not increase the incidence of acute urolithiasis with the presence of urinary calculi. A description of calculi and urolithiasis in mink was presented previously (Chapter 3).

Radiographs showed different cardiac conditions in the mink such as cardiomegaly and microcardia. One mink had an enlarged right aortic arch on the initial radiograph, which appeared normal thereafter. However, adult female mink receiving the diet containing PCB 126 showed a significant reduction in mean heart weight compared to the control group (Chapter 3). In support of that finding, Hochstein et al. (1998) also observed a significant decrease in mean heart weight in surviving mink exposed to 1 μg TCDD/kg feed in their diet compared to the control mink. The mechanism resulting in this effect is unclear.

CONCLUSIONS

Mink exposed to PCB 126 exhibited a neoplastic bone lesion characterized by osteolysis of the mandible and maxilla. However, the effects of PCB 126 on bone were not manifested systemically in mink, and no changes in mineral analyses were observed. Based on the current mink studies, the observed osteolysis of maxillae and mandibles is similar to the lesion that has been reported in other wildlife species exposed to environmental contaminants. This neoplastic lesion in mink exposed to PCB 126 may also be similar to the oral lesions that have been reported in humans from Yusho, Japan and Taiwan. However, PCB 126 apparently does not act similarly in mink as it does in rats and mice (Lind 1999). Therefore, when performing comparative studies it is imperative to consider the possibilities of species differences, especially when extrapolating studies to other species, such as humans in a risk assessment situation.

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CHAPTER 6

Squamous Epithelial Lesion of the Mandibles and Maxillae of Wild Mink (*Mustela vison*) Naturally Exposed to Polychlorinated Biphenyls.

K.J. Beckett, S.D. Millsap, A.L. Blankenship, M.J. Zwiernik, J.P. Giesy and S.J. Bursian

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Squamous Epithelial Lesion of the Mandibles and Maxillae of Wild Mink (*Mustela vison*) Naturally Exposed to Polychlorinated Biphenyls.

ABSTRACT

Approximately 125 km of the Kalamazoo River, located in southwestern Michigan, USA, are designated as a Superfund site with polychlorinated biphenyls (PCBs) as the contaminant of concern. Mink (*Mustela* vison) are a naturally occurring predator in this area, and also a species of concern due to their known sensitivity to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and structurally similar compounds such as PCBs. Four of nine mink trapped from the Kalamazoo River area of concern (KRAOC) exhibited histological evidence of a jaw lesion previously identified in ranch mink. The jaw lesion, proliferation of squamous epithelium in the mandible and maxilla, is known to be caused by 3,3',4,4',5-pentachlorobiphenyl (PCB 126) and TCDD. Mink trapped from an upstream reference area (Fort Custer Recreation Area, FCRA) did not exhibit the lesion. Mean concentrations of total PCBs were 2.8 and 2.3 mg/kg, wet wt in the livers of mink from the KRAOC and FCRA, respectively, and TCDD toxic equivalent (TEQ) concentrations were 0.30 and 0.11 μg TEQs/kg, wet wt, respectively. There were significant correlations between the severity of the lesion and hepatic concentrations of total PCBs and TEQs. This is the first published report of the lesion occurring in wild mink.

INTRODUCTION

The Kalamazoo River Superfund site is located in southwestern Michigan, USA and encompasses approximately 125 km (80 miles) of the Kalamazoo River, from Morrow Dam in Kalamazoo County to Lake Michigan. The Kalamazoo River area of concern (KRAOC) became contaminated with polychlorinated biphenyls (PCBs) by waste discharged from the recycling and processing of carbonless copy paper where the primary source of the PCBs was Aroclor 1242 and to a lesser degree Aroclor 1254 (Camp et al. 1999).

Mink are a naturally occurring predator in the Kalamazoo River area, and also a species of concern due to their known sensitivity to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and compounds of similar structure such as PCBs (Aulerich and Ringer, 1977; Aulerich et al. 1985; Hochstein et al. 1988; Heaton et al. 1995; Hochstein et al. 1998; Pastva 2003). To assess the possible effects of PCBs on wildlife residing in the Kalamazoo River basin, wild mink were trapped from two areas, the KRAOC and the Fort Custer Recreation Area (FCRA), a reference area with lesser concentrations of PCBs (Pastva 2003).

The potential for adverse effects of PCBs on mink in the KRAOC was estimated using two approaches, by applying dietary exposure models and by measuring liver concentrations of TCDD toxic equivalents (TEQs) and total PCBs in trapped mink (Pastva 2003). The concentrations of total PCBs and TEQs measured in the livers of the wild mink were in the range of those of ranch mink that had expressed proliferation of

squamous epithelium in the mandibles and maxillae when exposed to PCBs in the diet (Bursian et al. 2003).

Previous studies have indicated that ranch mink of different ages exposed to 24.0 μg 3,3',4,4',5-pentachlorobiphenyl (PCB 126)/kg feed or 2.4 μg TCDD/kg feed developed clinical signs of mandibular and maxillary squamous epithelial proliferation that in severe cases resulted in the loss of teeth (Render et al. 2000*a*; 2000*b*; 2001). In an unpublished study (K. Beckett, Michigan State University, East Lansing, MI), we have shown that mink fed diets containing concentrations as low as 0.24 μg PCB 126/kg developed the clinical lesion, although the required period of exposure was longer. The lesion develops when squamous epithelial cells form infiltrating nests and cords into the periodontal ligament and alveolar bone, which results in osteolysis. Infiltration of squamous cells into the periodontal ligament eventually causes loose and displaced teeth. The maxillae and mandibles became markedly porous due to loss of alveolar bone, with concomitant loss of teeth, leading, in severe cases, to aphagia (the inability to eat).

The purpose of this study was to determine if wild mink inhabiting a PCB-contaminated environment exhibited squamous epithelial lesions of the mandibles and maxillae that until now have been reported only in ranch mink exposed to PCB 126 or TCDD under laboratory conditions.

METHODS

Wild mink were trapped throughout the KRAOC and the upstream reference area, FCRA, during the winters of 2000, 2001, and 2002 (Pastva 2003). Nine male mink were collected within the KRAOC from D Avenue downstream to the former Trowbridge impoundment, a distance of approximately 25 river km. Three mink (two males and one female) were collected from the upstream reference area at Fort Custer, approximately 25 river km upstream of D Avenue, in Kalamazoo, MI, USA. Frozen mink carcasses were brought to the laboratory and thawed. A gross necropsy was performed by a board-certified veterinary pathologist. The weight (without pelt) and length (nose to base of tail) of each mink were determined. Organ condition was assessed during necropsy to determine if there were gross abnormalities. Samples of the livers were collected for histological assessment, as well as for determination of total PCB and TEQ concentrations.

For chemical analysis, polychlorinated biphenyls were extracted from liver tissue based on U.S. Environmental Protection Agency method 3540 (Soxhlet extraction) (U.S. EPA 1996). An aliquot of the extract was analyzed for total PCBs with a gas chromatograph (Perkin Elmer Autosystem, Boston, MA, USA) equipped with a ^{63}Ni electron capture detector according to Nakata et al. (1998). A second aliquot of the extract was subjected to carbon column chromatography for the separation of non-*ortho*-substituted (coplanar) PCB congeners. The resulting extracts were analyzed by gas chromatography-mass spectroscopy on a Hewlett Packard 5890 Series II gas

chromatograph (Rolling Meadows, IL, USA) equipped with a Hewlett Packard 5972 series mass selective detector (Pastva 2003).

The toxic equivalency method relates the toxicity of TCDD-like chemicals including certain polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and PCBs to the toxicity of TCDD, which is considered the most toxic of this group of chemicals. In this study, TEQs were calculated by multiplying the concentration of an individual PCB congener by its toxicity equivalency factor (TEF) as presented in Van den Berg et al. (1998). The TEF denotes a congener's toxicity relative to TCDD, which has a toxicity value of 1.0. Total TEQ concentrations in liver samples were determined by summing the concentrations of TEQs of individual congeners detected in each sample. PCB congeners 77, 81, 105, 118, 126, 156, 157, 167 and 169 were used for the calculation of TEQs (Pastva 2003).

In all cases, heads were removed from carcasses and fixed in 10% neutral-buffered formalin. Heads were then decalcified in Surgipath® Decalcifier II (Surgipath Medical Industries, Richmond, IL, USA) and trimmed, and the mandibles and maxillae collected. Jaws were processed for histological examination, sectioned to 5 μm, stained with hematoxylin and eosin, and the slides examined using light microscopy.

Lesions, the development of which is a dynamic process, were rated from mild to severe based on the following criteria: a rating of "mild" is characterized by one or a few small, focal cyst(s), which is the initial phase of the lesion; a rating of "moderate" is characterized by the presence of several foci of cysts or islands of squamous epithelial cells, or a few larger cysts, that invade the periodontal pockets and disrupt the teeth; a rating of "severe" is assigned when cysts, increasing in size and invasiveness, infiltrate

throughout the mandible and/or maxilla, destroying all surrounding tissues including the alveolar bone that supports tooth structure. Correlation between total PCB and TEQ concentrations in the liver and the severity of the lesion (numerically scored from one to three based on the above rating description) was assessed by the Pearson correlation analysis using SAS PROC CORR (Statistical Analysis System, Release 8.0, Cary, NC, USA).

RESULTS

Mink from both the KRAOC and FCRA areas appeared to be healthy based on body weights, gross examination and histological assessment of liver tissue. However, four of the nine mink from KRAOC had histological evidence of mandibular and maxillary squamous epithelial proliferation (Figure 6-1), while none of the mink in the FCRA had the lesion. The severity of the lesion ranged from mild to moderate (Table 6-1).

Total concentrations of PCBs in the livers of the four mink from the KRAOC that expressed the lesion ranged from 2.9 to 6.0 mg/kg, wet wt. Total PCB concentrations in livers of the other five mink from the KRAOC ranged from 0.05 to 3.4 mg/kg, wet wt. Concentrations of TEQs in the livers of the four mink from KRAOC that exhibited the lesion ranged from 0.21 to 1.3 μg/kg, wet wt and from 0.02 to 0.25 μg/kg, wet wt in the five mink not expressing the lesion (Table 6-1). Concentrations of total PCBs in the livers of the three mink from the FCRA ranged from 1.6 to 3.7 mg/kg,

wet wt, while concentrations of TEQs ranged from 0.05 to 0.20 μg/kg, wet wt (Table 6-1). PCB 126 is known to be a potent inducer of squamous proliferation of the mandibles and maxillae (Render et al, 2000*a*; 2000*b*; 2001). In this study, the primary PCB congener contributing to the TEQ concentrations in livers of the mink trapped in the Kalamazoo River Basin was PCB 126 with an average contribution of 77% at KRAOC and 69% at FCRA (Table 6-1).

Lesion severity was significantly and positively correlated with both concentrations of total PCBs and TEQs in livers of mink collected from the KRAOC. Correlation coefficients (r^2) were 0.88 ($p < 0.001$) and 0.89 ($p < 0.001$) for total PCBs and TEQs, respectively.

DISCUSSION

When six- and 12-week-old ranch mink kits were exposed to 2.4 μg TCDD or 24.0 μg PCB 126/kg feed, they displayed gross displacement of incisor and canine teeth with nodular swelling of the mandibular and maxillary gingiva within four weeks of exposure. The PCB 126- and TCDD-induced lesion reported in these initial studies was characterized by proliferation of squamous epithelial cells that formed infiltrating nests and cords into the periodontal ligament and the alveolar bone causing osteolysis. The maxillae and mandibles became markedly porous due to loss of alveolar bone, with concomitant loss of teeth, which led to aphagia and rapid weight loss (Render et al. 2000*a*; 2000*b*; 2001).

The lesion subsequently was reported to develop by 24 weeks of age in ranch mink kits exposed to 0.24 μg PCB 126/kg feed *in utero* through sexual maturity (K. Beckett, Michigan State University, East Lansing, MI). Histological evidence of proliferation of squamous epithelial cells in the mandibles and maxillae was also observed in adult mink fed a diet containing 0.24 μg PCB 126/kg feed (K. Beckett, Michigan State University, East Lansing, MI), which is an environmentally relevant concentration (Hochstein et al. 1988; Tillitt et al. 1996; Bursian et al. 2003). Hepatic concentrations of PCB 126 and TCDD were not measured in the initial jaw lesion studies by Render and associates (Render et al. 2000*a*; 2000*b*; 2001).

Concentrations of total PCBs and TEQs in the liver were measured in a recent study where ranch mink fed diets containing PCB-contaminated fish collected from the Housatonic River in Massachusetts, USA developed the jaw lesion (Bursian et al. 2003). In that laboratory study in which mink were exposed to 1.6 mg total PCB/kg feed (0.02 μg TEQs/kg feed) from conception through 31 weeks of age, the lesion was detected at a similar frequency as reported in the present study. The average concentrations of total PCBs and TEQs in the livers of mink fed the diet containing Housatonic River fish were 3.5 mg/kg, wet wt and 0.10 μg/kg, wet wt, respectively. Of the mink trapped in the KRAOC, 44% had histological evidence of mandibular and maxillary squamous epithelial proliferation. The average concentrations of total PCBs and TEQs in the livers of KRAOC mink exhibiting the lesion were 4.3 mg/kg, wet wt and 0.60 μg/kg, wet wt, respectively. The average total PCB concentration in the livers of the KRAOC mink is comparable to the value reported by Bursian et al. (2003) in

Figure 6-1. Mandibular and maxillary squamous epithelial hyperplasia in wild mink exposed to polychlorinated biphenyls in the Kalamazoo River area of concern (KRAOC). **A**: A section from a control mink is shown for reference. Note the solid-appearing alveolar bone (AB) surrounding the teeth (T), which is above the gingiva (GIN). **B**: KRAOC Mink #03 had a lesion rating of mild, with one focus (arrow). **C**: KRAOC Mink #06 had a lesion rating of mild-moderate, with a large invasive cyst (arrow) and small multi-focal diffuse nests. **D**: KRAOC Mink #08 had a lesion rating of moderate, with diffuse squamous cysts throughout the jaw (arrows). Extensive infiltration by large islands of squamous epithelial cells has occurred and formed cysts that resulted in the loosening of teeth. These cysts may contain centers of exfoliated epithelia and sometimes keratin.

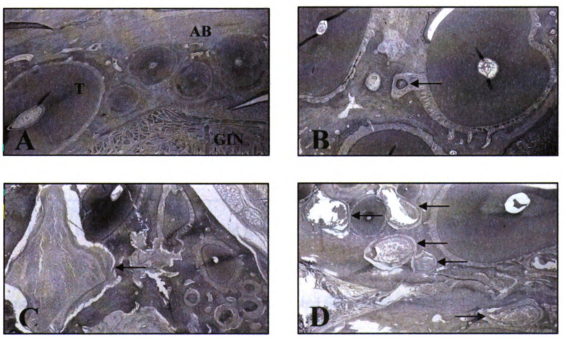

Table 6-1. Incidence of mandibular and maxillary squamous hyperplasia, and hepatic concentrations of total polychlorinated biphenyls (PCBs), toxic equivalents (TEQs) and percent contribution toward toxic equivalents by 3,3',4,4',5-pentachlorobiphenyl (PCB 126) in wild mink trapped in the Kalamazoo River Basin[a].

Mink #	Trapping Location	PCBs (mg/kg, wet wt)	TEQs (µg/kg, wet wt)	PCB 126 (%)	Lesion Rating
08	KRAOC	6.0	1.3	91	Moderate
06	KRAOC	5.0	0.58	90	Mild-moderate
02	KRAOC	2.9	0.33	83	Mild
01	KRAOC	3.4	0.25	86	No lesion
03	KRAOC	3.3	0.21	72	Mild
07	KRAOC	1.0	0.03	68	No lesion
09	KRAOC	0.05	0.03	99	No lesion
11	KRAOC	1.6	0.03	0	No lesion
25	KRAOC	1.1	0.02	26	No lesion
		2.8 ± 0.7	***0.30 ± 0.14***		
1002	FCRA	3.68	0.20	80	No lesion
1003	FCRA	1.55	0.08	59	No lesion
1001	FCRA	1.59	0.05	68	No lesion
		2.3 ± 0.7	***0.11 ± 0.04***		

[a] KRAOC refers to the contaminated Kalamazoo River area of concern. FCRA refers to the Fort Custer recreation area, which is the upstream reference site. Data for total PCB and TEQ concentrations in the liver are presented for individual animals and as the mean ± standard error of the mean (*in italics*) for each of the two sites.

mink exposed to PCBs through consumption of fish collected from the Housatonic River (3.5 mg PCB/kg, wet wt). Concentrations of TEQs in the livers of mink from the KRAOC were six-fold greater than those in the livers of mink fed diets containing fish from the Housatonic River (0.10 μg/kg, wet wt). Hepatic concentrations of total PCBs and TEQs in livers of mink fed Housatonic River fish were determined in a pooled sample that was comprised of tissue from all animals in the group, regardless of lesion status. This could explain the difference in TEQ concentrations between these two studies. It is possible that hepatic concentrations of TEQs in only those mink on the feeding study exhibiting the lesion were greater than the group average (0.10 μg/kg, wet wt) and closer to the average reported for the KRAOC mink that had the lesion.

There was one mink each from the KRAOC and FCRA that did not develop the lesion (Table 6-1), yet had concentrations of total PCBs and TEQs in the liver that would be expected to be associated with the expression of mandibular and maxillary squamous epithelial hyperplasia. One explanation is individual variation in sensitivity to TCDD-like chemicals. In addition, the duration of exposure, the age during exposure and/or the time required for the lesion to develop could have been suboptimal for its manifestation in these individuals.

CONCLUSIONS

In conclusion, the data presented here indicate that wild mink trapped in the Kalamazoo River area of concern exposed to environmentally-derived PCBs developed

the lesion characterized as mandibular and maxillary squamous epithelial hyperplasia. The average concentrations of total PCB and TEQ in livers of mink trapped from KRAOC that expressed the lesion were 4.3 mg/kg, wet wt and 0.60 μg/kg, wet wt, respectively. There was a positive correlation between hepatic total PCB and TEQ concentrations and the presence and severity of the jaw lesion observed in wild mink. Therefore, mink exposed to relevant concentrations of TCDD-like compounds could experience increased severity of the lesion leading to erosion of the mandibles and maxillae with concomitant loss of teeth, and eventually aphagia. This jaw lesion, now identified in a wild mink population, poses a threat to wildlife health and survival. The results of this study are consistent with the predicted hazard quotients based on concentrations of both total PCBs and TEQs in the liver and effects on survival and weight gain in mink kits whelped of mink exposed to these compounds in the diet. However, even though the lesion was observed in this population of mink, the body conditions of the mink did not indicate that it was resulting in effects on body weight (Pastva 2003). These results indicate that the occurrence of the lesion in mink is a sensitive, functional measure of exposure to PCBs in the diet, but may not translate into population level effects. This may be due to several compensatory factors, including sufficient reproductive capacity to offset adverse effects on the longevity of individual mink or the fact that the lesion does not become sufficiently severe during the normal lifetime of mink living in the wild.

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